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14. ABSTRACT
In breast cancer progression, the transition from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) is a life-threatening step. This step is accompanied by a dramatic drop in prognosis. Stromal fibroblasts and epithelial cells are separated by a basement membrane (BM) at the early DCIS stage. However, once the BM is disrupted and stromal invasion of epithelial cells is initiated, direct heterocellular contacts between fibroblasts and epithelial cells often occurs. This suggests that the signaling through the heterocellular contact may be a crucial factor in how the invasive progress continues after it is initiated. Accordingly, the objective of this proposal is to investigate the influence of heterocellular contacts between MCF-DCIS cells and human mammary fibroblasts (HMFs) in breast cancer progression by employing a microfluidic-based compartmentalized 3D co-culture platform enabling both contact-free and contact-associated co-cultures.

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Introduction

In breast cancer progression, the transition from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) is a life-threatening step. This step is accompanied by a dramatic drop in prognosis. Stromal fibroblasts and epithelial cells are separated by a basement membrane (BM) at the early DCIS stage. However, once the BM is disrupted and stromal invasion of epithelial cells is initiated, direct heterocellular contacts between fibroblasts and epithelial cells often occurs. This suggests that the signaling through the heterocellular contact may be a crucial factor in how the invasive progress continues after it is initiated. Accordingly, the objective of this proposal is to investigate the influence of heterocellular contacts between MCF-DCIS cells and human mammary fibroblasts (HMFs) in breast cancer progression by employing a microfluidic-based compartmentalized 3D co-culture platform enabling both contact-free and contact-associated co-cultures. Here we report progress to date including the completion of Specific Aim 1. We are on track to complete Specific Aim 2 within the approved no cost extension period.

Body

Building our preliminary data(*1*), we accomplished Task 1 (development of a microscale culture platform) and Task 2 (validation of heterocellular contact formation between MCF-DCIS cells and HMFs in contact-associated co-cultures) listed under Specific Aim 1. Moreover, by using the microscale compartmentalized 3D co-culture platform, we verified that the HMFs near the MCF-DCIS cells were activated showing a few protrusions. The actin-rich protrusions in HMFs were correlated with local concentration increase of beta-1 integrins suggesting that the protrusions express sticky ends that stimulate adhesion of HMFs to surrounding extracellular matrices as well as to other cells. In addition, we analyzed soluble molecules secreted by HMFs cultured in 2D and 3D conditions and found that the HMFs in a 3D condition secreted higher concentration of a few molecules such as HGF, CXCL12, and MMP14. The HMFs in a 3D condition have higher impact on DCIS progression to IDC. Currently, we are exploring mechanisms involved in the activation of fibroblasts in co-cultures with MCF-DCIS cells, and will proceed to identify junction molecules between MCF-DCIS cells and HMFs.

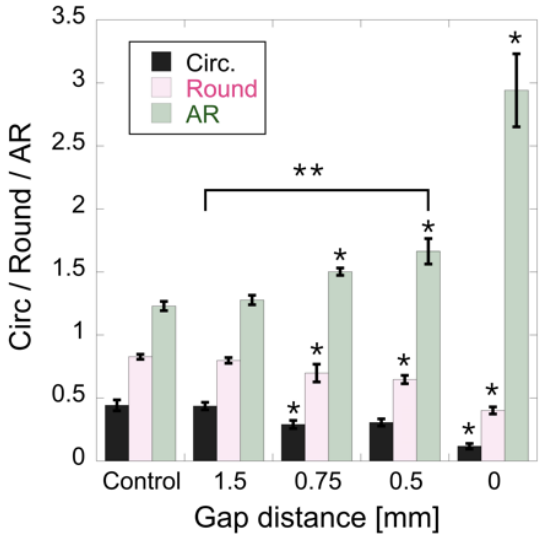

Fig. 1: Bar graph presents the values from shape descriptor analysis of MCF-DCIS cells, showing decreasing circularity and roundness and increasing aspect ratio as gap distance decreases. Error bars represent standard error. *: $p<0.005$ compared with the control group. **: $p<0.0005$ compared with gap '0' group.

1) Development of a microfluidic-based, compartmentalized co-culture platform to investigate the differences between contact-assisted and contact-free transitions (Specific Aim 1, Task 1):

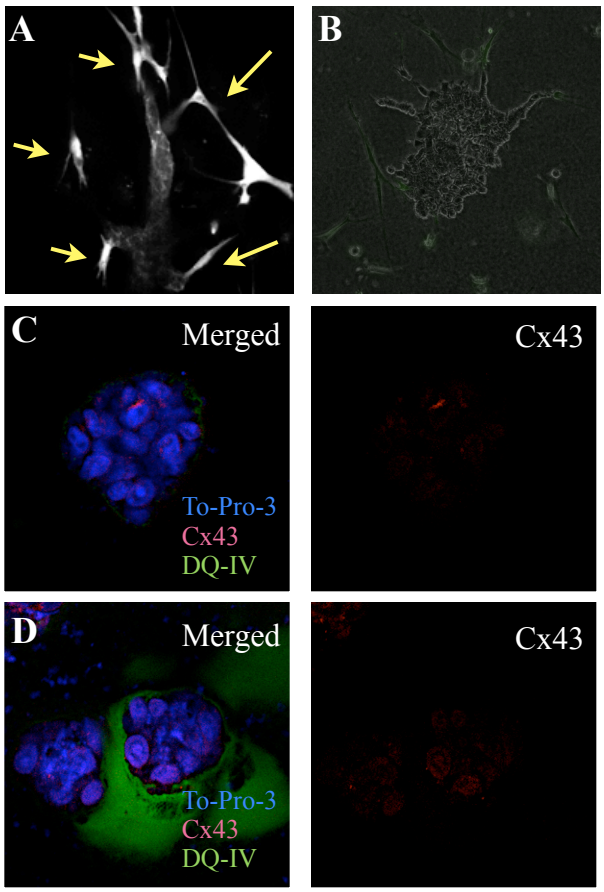

Fig. 2: (A) Invasive MCF-DCIS cells showing multiple contacts with HMFs (yellow arrows). (B) MCF-DCIS cluster co-cultured with HMFs (shown in green). (C) Non-invasive MCF-DCIS cluster showing Cx43 expressions at cell junctions. (D) Invasive MCF-DCIS clusters showing internalized Cx43 expressions. Blue:To-Pro-3, Red: Cx43, Green: DQ-collagen IV

Each cell type was prepared in a mixed matrix and co-injected into the 'Y' shaped channel to form separate but adjacent compartments and cultured for longer than 9 days. Interestingly, MCF-DCIS cells near the interface showed invasive growth (Circularity (Circ): 0.21±0.11, Roundness (Round): 0.54±0.22, Aspect ratio (AR): 2.29±1.23), while the cells upstream, before the two channels join (>1.5 mm from the interface), retained rounded cluster morphology (Circ: 0.47±0.17, Round: 0.79±0.13, AR: 1.3±0.29) providing a built-in monoculture control. This distance dependency was further distinguished by inserting a blank spacer gel (same ECM composition) of specific width (i.e., 0, 0.5, 0.75, and 1.5 mm) between the two cell-containing compartments. These configurations were achieved by preparing microchannels of three or four input ports. The spacer gel minimized direct contact of MCF-DCIS and HMF cells, but still allowed diffusion of molecules. After 7 days of culture, morphological differences between control (mono-culture), contact co-culture (no spacer) and co-culture with spacer gel were observed. Specifically, based on a shape descriptor (Circ, Round, and AR) analysis shown in a bar graph in Fig. 1, a clear invasive transition was observed in contact co-culture, a short protrusive morphology was observed when a gel spacer separated the two cell types and maintenance of rounded clusters was observed in mono control (either at sufficient distance from the fibroblast compartment or in a separate channel). In order to examine any possible microfluidic system specific influence, transwell (liquid spacing) and layered gel (gel spacing) co-cultures were also performed and similar trends were observed.

2) Validation of heterocellular contacts formed between MCF-DCIS cells and HMFs in adjacent co-culture (Specific Aim 1, Task 2):

In the adjacent co-culture condition, MCF-DCIS cells at the interface showed more invasive transition with multiple heterocellular contacts with nearby HMFs (Fig. 2A). The heterocellular contacts were verified by performing F-actin staining of MCF-DCIS cells and HMFs. By performing mixed co-cultures of MCF-DCIS clusters and HMFs in a 3D condition, it was observed that HMFs formed contacts with MCF-DCIS clusters and positioned at the leading

edge of MCF-DCIS clusters (Fig. 2B). McLachlan et. al. have shown that connexins are tumor suppressors, and human breast connexin 26 (Cx26) and connexin 43 (Cx43) gap junctions are often down-regulated in breast cancer(*2*). Accordingly, we performed immunofluorescent staining of human Cx26 and Cx43 and compared the signal intensities of the two connexins. Dye-quenching(DQ) Collagen IV was embedded in the collagenous matrix to visualize the degree of matrix degradation caused by MCF-DCIS cells (i.e., higher intensity of DQ collagen IV is expressed by higher invasion of MCF-DCIS cells). As shown in Fig. 2C and 2D, Cx43 molecules were concentrated at the junctions of non-invasive MCF-DCIS cells (mono-cultured), and they were internalized in invasive MCF-DCIS cells (co-cultured with HMFs). Although these images showed promising data, it is still challenging to obtain reliable, repeatable data from these immunofluorescent signal analyses due to the non-specificity of antibodies and background signals from matrices and other molecules. Hence, we plan (as part of Specific Aim 2) to knockdown or inhibit the expressions and functional activities of a few connexins as alternative approaches.

3) Investigation of fibroblasts' activation caused by the proximity to MCF-DCIS cells:

We are also exploring how the HMF become activated and protrusive when co-cultured with MCF-DCIS cells, and to understand the biological function and impact of protrusive HMF during DCIS progression to IDC. This study demonstrates the one possible route through which DCIS cells trigger the activation of pre-existing fibroblasts and subsequently, leads to the modification of the ECM and the progression to IDC. We first demonstrate that the activation of fibroblasts is the result of a paracrine interaction in a microfluidic point-source gradient device. The microfluidic platform was used to thoroughly characterize the distinctive morphology change (i.e., increased protrusions) in HMFs when they are exposed to the secretome produced by MCF-DCIS cells (Fig. 3). Secondly, we developed novel imaging analysis algorithms to quantify the morphology of HMFs, the protein localization within them, and the alterations in the surrounding ECM. Using the analysis algorithms, we verified that the intensities of F-actin and beta-1 integrins were co-localized along the edge of protrusive HMFs suggesting the sticky ends of HMFs (Fig. 3). Next, we verified that the signaling based on Cathepsin D (CTSD) produced from MCF-DCIS and low-density lipoprotein receptor-related protein-1 (LRP1) in HMFs was responsible for the protrusive activity of HMFs. We also established that MCF-DCIS cells expressed cathepsin-D at a higher level than several other mammary epithelial cell lines, and that the degree of phenotype alteration in HMFs is dependent on the amount of cathepsin-D present in the local environment.

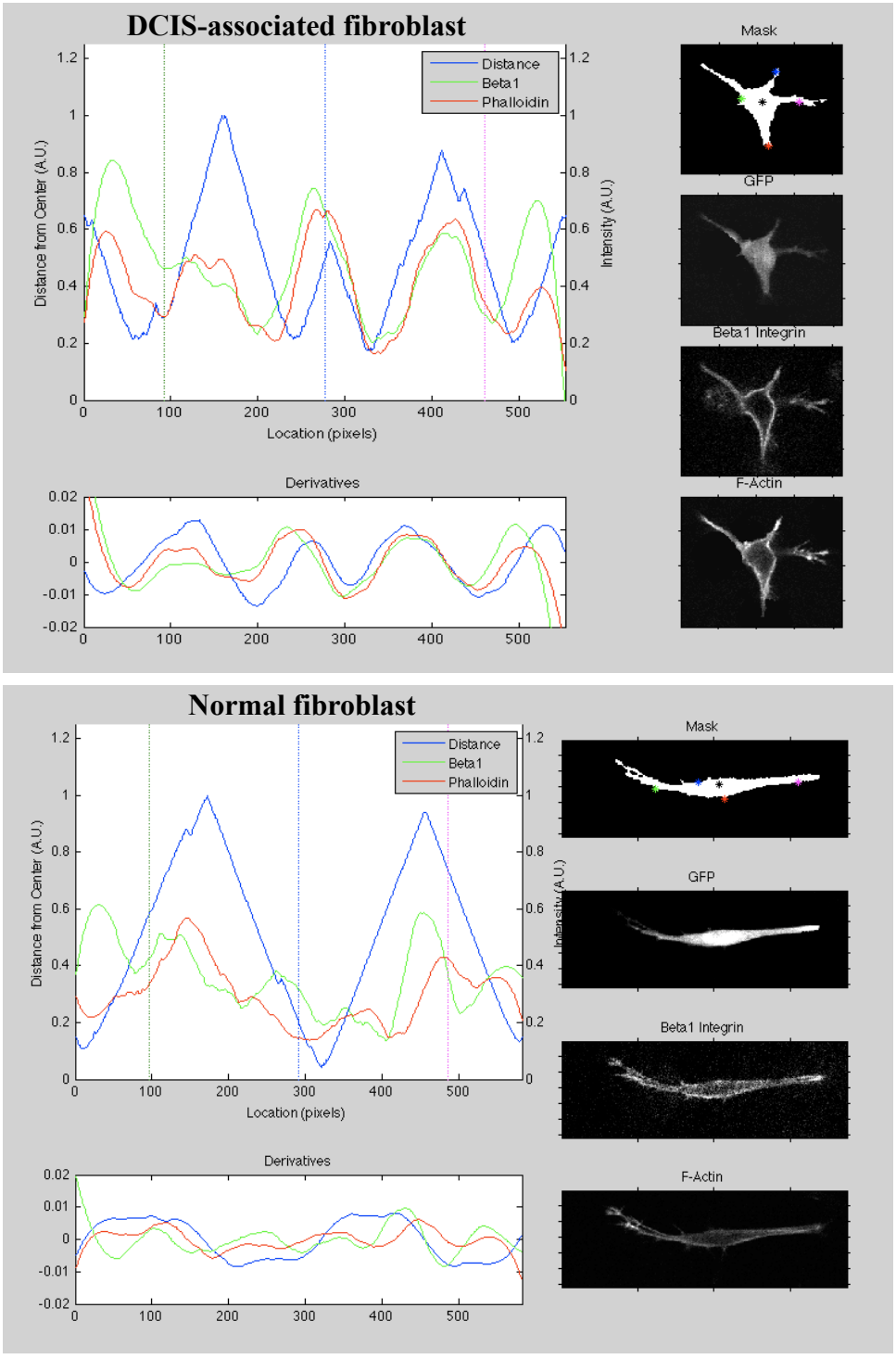

Fig. 3: DCIS cells activate fibroblasts from normal fibroblasts (NF) to DCIS associated fibroblasts (DF) (showing more protrusion and higher concentration of F-actin). The distance from center of mass (red dot shown in image masks) was measured to profile morphologies of NF and DF (shown in blue). The intensities of F-actin (red) and beta-1 integrin (green) were estimated along the cell membrane. DF showed multiple F-actin and beta-1 integrin rich protruding edges, while NF showed relatively homogeneous F-actin and beta-1 integrin distribution along the cell membrane.

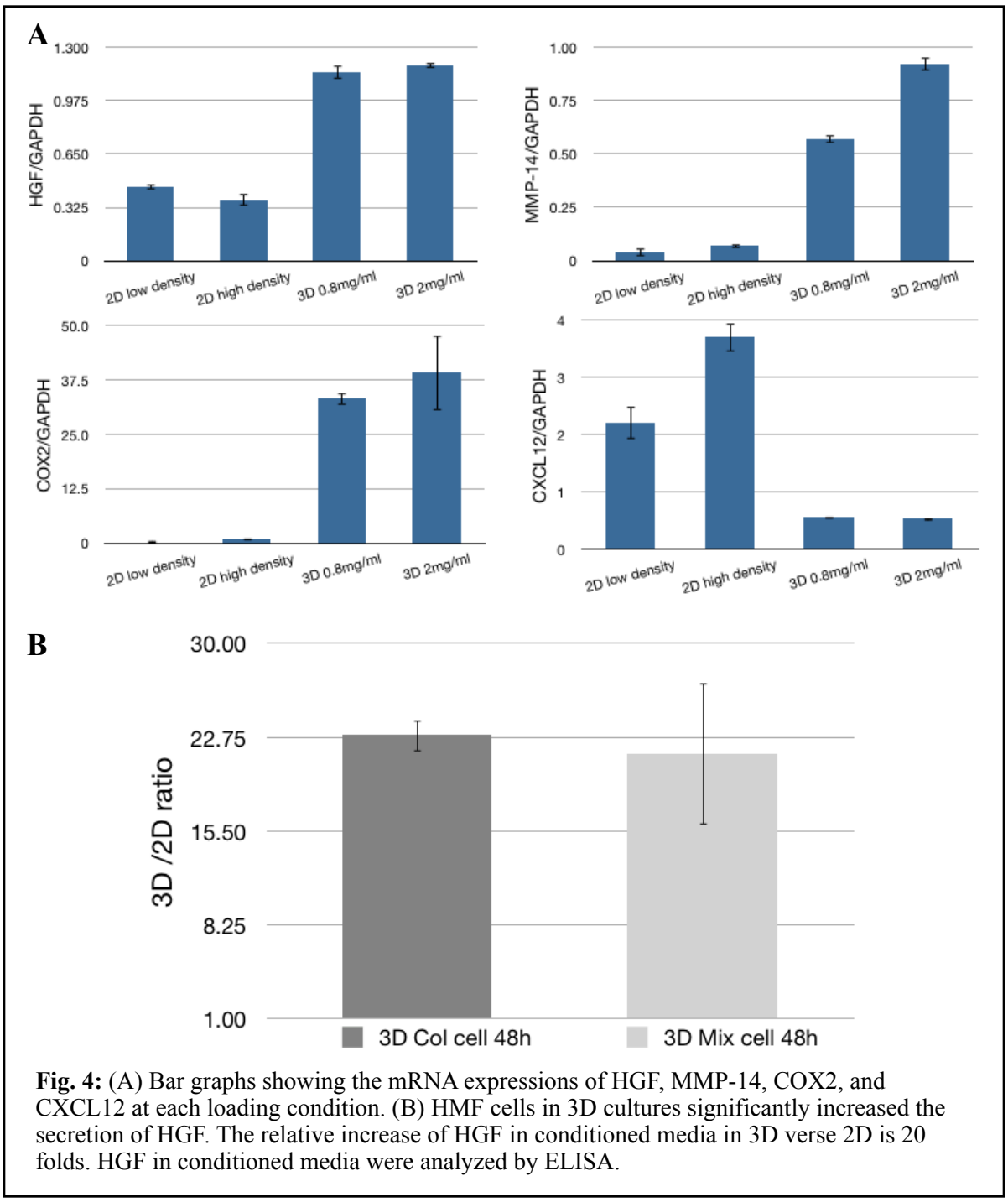

Fig. 4: (A) Bar graphs showing the mRNA expressions of HGF, MMP-14, COX2, and CXCL12 at each loading condition. (B) HMF cells in 3D cultures significantly increased the secretion of HGF. The relative increase of HGF in conditioned media in 3D verse 2D is 20 folds. HGF in conditioned media were analyzed by ELISA.

4) Exploration of different functional activities of fibroblasts in 3D and 2D conditions:

While it is generally accepted that 3D systems are more physiologically relevant, we still need clearer evidence why and how a 3D system makes a difference by directly comparing functional behaviors in 2D versus 3D systems. In this study, we directly compare functional behaviors of HMFs cultured in 2D and 3D conditions and their effects on the invasive progression of MCF-DCIS cells by using both traditional macro scale culture systems and

emerging micro scale culture models. We adopted a known mechanism in the invasive progression of breast cancer, such as the HGF and c-Met interaction to present the different regulatory behaviors of the interaction in different culture conditions(*3*, *4*). We first observed that conditioned media from 3D culture of HMFs induced increased invasion of MCF-DCIS cells. Additionally, 3D culture increased the expression of three stroma-derived molecules (MMP14, HGF, and COX2) (Fig. 4A). HMFs in 3D culture produced a twenty-fold increase in HGF concentration as compared to 2D culture (Fig. 4B). The role of HGF was validated by neutralizing the activity of HGF as well as by inhibiting the phosphorylation of c-Met (cognate receptor of HGF). No difference in HGF expression was observed with different matrix compositions nor was HGF expression affected by blocking the function of β1 integrin suggesting a structural/mechanical mechanism may be involved in upregulating HGF in 3D culture conditions. Importantly, we developed a microfluidic in vitro platform that allows combined 3D and 2D co-cultures. The platform facilitates simultaneous endpoints of each cell compartment, reduces the number of cells and ECM proteins required and reduces experimental bias by allowing the simultaneous co-culture of cells in 2D and 3D laying the foundation for a more efficient and complete investigation of the complex interactions at play.

Key research accomplishments

1. Microfluidic 3D compartmentalized co-culture platform was developed that enabled investigation of distance-dependent invasive transition of MCF-DCIS cells.
2. By using the developed microfluidic platform, we revealed that the distance between cancer cells and fibroblasts is an important factor in stimulating invasive transition of MCF-DCIS cells.
3. Heterocellular contacts between cancer cells and fibroblasts were identified as MCF-DCIS cells progressed to invasive phenotype through F-actin visualization. These contacts further stimulated the invasive transition of MCF-DCIS cells.
4. The compartmentalized microfluidic co-culture platform enabled to observe different characteristics of HMFs in the proximity to MCF-DCIS cells.
5. We found that HMFs in a 3D condition secreted a few paracrine signaling molecules such as HGF, COX2, and MMP14 at higher concentration compared to HMFs in a 2D condition, and the increased secretion of the molecules accelerated the invasive transition of MCF-DCIS cells.

Reportable outcomes

1. Submitted manuscripts:

- Sung K E, Su X, Pelhke C, Berthier E, Friedl A, Beebe DJ, “Deciphering the role of microenvironment in regulating invasion in breast cancer: Effect of 3D culture of fibroblasts on the secretion of hepatocyte growth factor”.
- Montanez-Sauri S I, Sung K E, Berthier E, Beebe DJ, “Enabling Screening in 3D Microenvironments: Probing the Matrix and Stromal Components Effect on the Morphology and Proliferation of T47D Breast Carcinoma Cells”.

2. Presentation:

- Sung K E, Pelhke C, Berthier E, Friedl A, Beebe DJ, "A micro scale in vitro model of the DCIS-IDC transition: Deciphering the role of the stroma fibroblasts", The 28th International Association for Breast Cancer Research/Breakthrough Breast Cancer Conference, Manchester, UK, 2012, Oral presentation

Conclusion

We have developed a simple compartmentalized 3D co-culture model that supports the DCIS to IDC transition in vitro. The model enabled us to study heterocellular-contact involved and contact-free invasive transition of MCF-DCIS cells by varying the distance between cancer and fibroblasts compartments. The ability to examine distance dependence uncovered potentially new insights about the transition to invasion suggesting the possibility of a two-step process via two different progression mechanisms: first, a soluble factor-based progression and, second, cell-cell contact signaling involved progression. Moreover, we are currently exploring alterations in fibroblasts near to MCF-DCIS cells to understand how fibroblasts are activated to form heterocellular contacts with MCF-DCIS cells as well as how the fibroblasts change the surrounding microenvironment.

These observations were made possible by the unique functionality of the microscale model and have important implications in guiding the way we think about the transition and the development of therapeutic approaches to inhibit transition. Importantly, the simplicity of the microfluidic system enables efficient investigation of the mechanisms involved in DCIS progression and allows screening approaches to identify pathways involved. For example, the small volumes required per endpoint open the door to the use of neutralizing antibodies or siRNA approaches. The flexibility of the system will allow it to be readily adapted to create relevant in vitro 3D models for other diseases where soluble factor signaling between different cell types is important.

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3. K. Lindemann *et al.*, Differential expression of c-Met, its ligand HGF/SF and HER2/neu in DCIS and adjacent normal breast tissue, *Histopathology* **51**, 54–62 (2007).

4. B. Cao *et al.*, Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models, *Proc Natl Acad Sci USA* **98**, 7443–7448 (2001).

Appendices

Attached separately

February 26, 2012

Editor, *Cancer Research*

I am enclosing a manuscript titled "Deciphering the role of microenvironment in regulating invasion in breast cancer: Effect of 3D culture of fibroblasts on the secretion of hepatocyte growth factor," co-authored with K.E. Sung, X. Su, C. Pehlke, E. Berthier, A. Friedl which we are submitting to *Cancer Research* for possible publication as a Research Article. We believe this paper would fit particularly well into the subcategory of Integrated Systems and Technologies.

This paper underscores the importance of 3D *in vitro* systems in paracrine interactions and identifies important factors that influence breast cancer progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) by directly comparing functional behaviors of human mammary fibroblasts (HMF) cultured in 2D and 3D conditions. One of our major findings was that HMFs in 3D culture produced a twenty fold increase in hepatocyte growth factor (HGF) concentrations as compared to HMFs in 2D culture. We also found that because of this increased HGF secretion, HMFs in 3D culture cause a more accelerated transition to IDC compared to 2D conditions. We also advanced *in vitro* system technology by developing a compartmentalized microfluidic *in vitro* platform that allows combined 3D and 2D co-cultures. This microfluidic system possesses several advantages over traditional co-culture systems. For example, our system facilitates simultaneous monitoring of each cell compartment and reduces the number of cells and ECM proteins required. This study will appeal to the diverse readership of *Cancer Research* because it presents intriguing findings in breast cancer progression and also presents technological advances in designing *in vitro* models.

Potential reviewers of this manuscript include:

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Please contact me if I can provide additional information about our manuscript. Thank you for considering it for possible publication.

Sincerely,

David J. Beebe
Professor

Deciphering the role of microenvironment in regulating invasion in breast cancer: Effect of 3D culture of fibroblasts on the secretion of hepatocyte growth factor

Kyung Eun Sung[1,2,3], Xiaojing Su[1,2], Carolyn Pehlke[1,2,3], Erwin Berthier[4], Andreas Friedl[2,3,5], David J. Beebe[1,2,3]

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Running title: The effect of 3D *in vitro* culture on cancer progression.

Key words: 3D system, Microfluidic system, Ductal carcinoma in situ, Tumor-stroma interaction, Breast cancer progression

Abstract

While it is generally accepted that 3D systems are more physiologically relevant, few studies directly compare cell behaviors in 2D and 3D conditions. Here, we directly compare functional behaviors of human mammary fibroblasts (HMFs) cultured in 2D and 3D conditions and their effects on the invasive progression of MCF10-DCIS.com (MCF-DCIS) cells. The studies were enabled by using both traditional macro scale culture systems and emerging micro scale culture models. We observed that conditioned media from 3D culture of HMFs induced increased invasion of MCF-DCIS cells. Additionally, 3D culture increased the expression of three stroma-derived molecules (MMP14, HGF, and COX2). HMFs in 3D culture produced a twenty-fold increase in HGF concentration as compared to 2D culture. The role of HGF was validated by neutralizing the activity of HGF as well as by inhibiting the phosphorylation of c-Met (cognate receptor of HGF). No difference in HGF expression was observed with different matrix compositions nor was HGF expression affected by blocking the function of β1 integrin suggesting a structural/mechanical mechanism may be involved in upregulating HGF in 3D culture conditions. Importantly, we developed a microfluidic *in vitro* platform that allows combined 3D and 2D co-cultures. The platform facilitates simultaneous endpoints of each cell compartment, reduces the number of cells and ECM proteins required and reduces experimental bias by allowing the simultaneous co-culture of cells in 2D and 3D laying the foundation for a more efficient and complete investigation of the complex interactions at play.

Introduction

Cells cultured in three-dimensions (3D), in which cells are completely embedded in extra cellular matrix (ECM), show differences in functional behaviors such as differentiation, proliferation, and gene expression, when compared to cells cultured in two-dimensions (2D) (i.e. monolayer on plastic) (1-3). With the increasing awareness of the limitations of 2D *in vitro* models, many researchers have shifted their focus to 3D *in vitro* models (3-6). 3D models can more faithfully recreate key aspects of the microenvironment and, in some cases, provide more comprehensive information, which is difficult to obtain from a 2D model (7-9). 3D *in vitro* environments alter cell signaling through enhanced interaction between cells and the surrounding ECM. Accordingly, different signaling mechanisms in 3D microenvironments, compared to 2D microenvironments have been increasingly reported over the last decade. For example, Fischbach et al. reported that 3D cultures of various breast cancer cell lines showed enhanced interleukin 8 (IL-8) secretions that depend on the engagement of integrins (10). Likewise, Cukierman et al. reviewed various 3D-dependent signaling events of fibroblasts with collagen gels and fibronectin-containing matrices that mimic the *in vivo* 3D microenvironment and generate adhesions comparable to 3D-adhesions *in vivo* (2).

Fibroblasts are implicated as key regulators of cancer development and progression. In an altered stroma surrounding a pre-invasive tumor, the fibroblasts become activated and can play a critical role in the progression to invasion via enhanced secretion of cytokines, growth factors, and proteases such as TGF-ß1, HGF, SDF-1, and MMP2 (11-13). Particularly in breast cancer, the progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) is believed to be actively driven by complex interactions with the surrounding microenvironment including the interactions with various stromal fibroblasts (14). Even though models of progression have been demonstrated using traditional *in vivo* animal models as well as *in vitro* models, commonly used *in vitro* systems such as co-cultures in transwells have limited functionality when investigating more complex mechanisms including paracrine/autocrine signaling, cell-cell physical interactions (homotypic and heterotypic) and matrix-cell interactions. We recently

developed an *in vitro* co-culture model of stromal and cancer cells that supports the progression from DCIS to IDC using a simple microfluidic system (15). Importantly, the microfluidic system is capable of manipulating the microenvironment more precisely than conventional systems enabling avenues of inquiry that are difficult to pursue using traditional systems. Our model was initially developed to investigate the influence of spatial separation between DCIS clusters and fibroblasts on the progression from DCIS to IDC. Here we utilize the system to explore the influence of 2D and 3D culture on the transition from DCIS to IDC and to enable functional screening of molecules involved in the progression mechanism.

Specifically, in this work we test the hypothesis that when fibroblasts are cultured in a 3D matrix, they secrete more paracrine signaling molecules than in 2D culture conditions and that these molecules induce a more invasive behavior in DCIS cells. While the importance of using 3D culture is well established, there are still relatively few studies directly comparing 2D vs. 3D *in vitro* systems. Hence, here we focus on direct comparisons of 2D and 3D cultures of human mammary fibroblasts (HMF) and their influence on cancer progression. We first collected different conditioned media from 2D and 3D cultures of HMF and explored the degree of invasive transition of MCF-DCIS cells in the different conditioned media. Second, we analyzed the mRNA expression of five stromal fibroblast-derived molecules (CXCL12, MMP14, HGF, COX2, and TGFß1) of HMFs cultured in 2D and 3D conditions. Among the five molecules, HGF was further investigated because of its known effect in the invasion of cancer cells. HGF is known to trigger cancer progression via activating Met on cancer cells. The HGF/Met signaling was further validated by adding a neutralizing antibody against HGF and a small molecule inhibitor that inhibits c-Met phosphorylation. Furthermore, we developed and applied a 3D microfluidic platform to perform 3D and 2D combined co-culture of MCF-DCIS cells and HMFs to validate the data obtained via conditioned media experiments. This work underscores the importance of a 3D microenvironment in paracrine interactions and identifies important factors that influence progression and whose expression is increased in 3D culture.

Results and Discussion

MCF-DCIS cells recapitulate key aspects of breast cancer progression from DCIS to IDC, and the progression is facilitated by co-existing human stromal fibroblasts. Previously, using a microfluidic 3D co-culture platform, we demonstrated that the MCF-DCIS showed invasive outgrowth when they were co-cultured with HMFs in a 3D mixed matrix (50:50 Matrigel:collagen 1 (0.8 mg/ml)), but they did not show significant invasive outgrowth when treated with conditioned media collected from a 2D culture of HMFs (15). Here, we assess the effects of 2D and 3D culture conditions on the secretion activity of HMFs. We first present the mRNA expression of stromal derived molecules to compare 2D vs. 3D culture of HMFs, and further investigate the effect of increased secretion of HGF from 3D cultured HMFs on the invasive progression of MCF-DCIS cells. Throughout this study, we employed traditional methods (i.e., 3D cultures in multiwells, transwell invasion assays, RT-qPCR, ELISA) to analyze gene expressions and protein concentrations and to estimate the degree of invasive transition in MCF-DCIS cells. We also designed a microfluidic 3D co-culture platform that allows combined 2D and 3D co-culture with short diffusion distances between cell types. The system allowed us to perform co-cultures of MCF-DCIS cells in a 3D condition with HMF cells in either 2D or 3D conditions. Traditionally, transwell systems have been used to perform combined 2D and 3D co-culture. However, these systems are limited in their ability to monitor the changes in both cell types in a single experiment, require relatively large numbers of cells, and significant quantities of expensive matrix proteins (e.g. collagen, matrigel). In addition, the surface areas and volume of the inserts in transwells (e.g., 0.3cm^2 and 0.2ml respectively in the 24-well format) are significantly smaller than the surface areas and volume of the bottom wells (e.g., 2.0cm^2 and 0.7ml respectively in the 24-well format), thus resulting in considerably different cell numbers and gel volumes between the bottom wells and the inserts. Properly designed microfluidic co-culture platforms overcome the limitations that conventional transwell systems possess. First, the small scale system requires 20 times fewer cells and reagents, saving money and increasing endpoints. Second, by properly designing the surface area of the microchannels, we can provide a similar surface area and sample volume for two different cell types in different culture

conditions. Third, by using the flow pinning method that stops a flow of sample solution at the designed position of microfluidic channels where there is a steep change of flow resistance, combined co-cultures of 2D and 3D conditions were enabled side-by-side, enhancing the capability of monitoring changes to both MCF-DCIS cells and HMFs with minimal inference from the other cell type (15).

Conditioned media collected from HMFs cultured in 3D induce a more invasive transition of MCF-DCIS cells than conditioned media collected from HMFs cultured in a 2D condition.

We first investigated the effect of conditioned media from HMFs cultured in 2D versus 3D conditions on the invasive progression of MCF-DCIS cells in 3D conditions. Conditioned media from 2D and 3D cultures of HMFs were collected after 48h cultivation in 48 well-plates and used to treat the 3D culture of MCF-DCIS cells in 48 well-plates. 3D conditioned media induced a more invasive transition of MCF-DCIS cells. We utilized several measures of invasion including the number of side branches and the aspect ratio in addition to traditional transwell invasion assays (i.e., the number of invaded cells). Treatment with 3D conditioned media resulted in more side branches as shown in Fig. 1A (45 vs. 12 in $120\mu m^2$). The aspect ratio (AR, major axis over minor axis) shows that the MCF-DCIS clusters cultured in 3D conditioned media (AR=1.32) were more elongated than the clusters cultured in 2D conditioned media (AR=1.44) (p=0.048) (Fig. 1B &C). Additionally, transwell invasion assays showed a higher invasion of MCF-DCIS cells when stimulated by 3D conditioned media than by 2D conditioned media (p=0.022) (Fig. 1D). To exclude any effect of Matrigel, which includes various soluble factors, similar experiments were performed with a collagen I matrix, and a similar trend was observed (Fig. 1D). These observations suggest that increased secretions of specific signaling molecules from fibroblasts occur in 3D conditions, and these consequently stimulate the invasive transition of MCF-DCIS cells.

To identify which molecules are secreted at higher concentrations in 3D conditions, we selected seven stroma-derived molecules (HGF, COX2, MMP2, MMP9, MMP14, TGF beta1, and CXCL12), and examined mRNA levels of five molecules (HGF, COX2, MMP14, TGF beta 1, and CXCL12) in HMFs cultured in 2D and 3D conditions. MMP2 and MMP9 secretions were analyzed by zymography. In the 3D conditions, two different collagen I concentrations (0.8 mg/ml and 1.6 mg/ml) of mixed matrix (50:50 Matrigel:collagen I) were used to explore the potential influence of different stiffness levels of the matrix, and the same cell loading density was prepared for both 3D conditions ($1.2x10^5$cells/well). For 2D conditions, we prepared two different cell loading densities (High, $6x10^4$cells/well; and Low, $3x10^4$cells/well) to examine whether different cell densities affect the level of secretion (Fig. 2A). HMFs proliferate faster under 2D conditions, so we used a lower loading density of 2D samples to obtain similar final cell densities in the 2D and 3D samples at the collection time (48 hours). The proliferation rate difference was measured and is shown in Fig. 2B. The mRNA expressions of the selected molecules were quantified after 48 hours cultivation. Among the five molecules tested, HGF, MMP-14, and COX2 showed higher expression when cultured in both 3D conditions than when HMF were cultured in 2D conditions. CXCL12 showed an opposite trend (Fig. 2C). TGF beta-1 did not show any significant difference between 2D and 3D cultures (data not shown). In addition, active MMP2 secretion was higher in HMFs cultured under 3D conditions, and MMP9 was not detectable in either 2D or 3D conditions (Fig. 2D).

These observations support our primary hypothesis that 3D *in vitro* culture of HMF activates secretion of paracrine signaling soluble molecules influencing the invasive transition of MCF-DCIS cells. For all molecules tested, no significant difference was observed with different cell and collagen densities. Thus, the 2D high density ($6x10^4$cells/well) and the lower concentration of the 3D matrix (0.8mg/ml) was used for all subsequent experiments. We further explored the influence of hepatocyte growth factor (HGF) on DCIS progression to IDC because HGF is a well-known scattering factor, a major contributor for invasive growth of cancer cells (16-18). Jedeszko et al., for example, showed that, using a conventional 3D *in vitro* model and an *in vivo* model, engineered HGF-secreting mammary fibroblasts increased the percentage of DCIS

structures with invasive outgrowth and activated c-Met (16). But this work did not compare the effect of fibroblasts cultured in 3D conditions and in 2D conditions on the scattering effect of DCIS in 3D in vitro systems.

Fibroblast-derived HGF production is increased in 3D *in vitro* culture and is necessary for progression of MCF-DCIS cells from a non-invasive to invasive phenotype.

HGF is a multi-functional cytokine stimulating invasion, motility, morphogenesis, as well as metastasis (19-22). Furthermore, over-expression of HGF has been detected in various invasive carcinomas, including breast carcinomas, and the high expression of HGF has been identified as a predictor of recurrence and shortened survival in breast cancer patients (17). HGF is known to act through its specific receptor, c-Met on cancer cells (23; 24). We measured HGF mRNA expression of MCF-DCIS cells in both 2D and 3D conditions, and the HGF mRNA expression was not detectable under any conditions examined (Fig. 3). Thus, in our model, main source of HGF paracrine signaling is from HMFs to MCF-DCIS cells.

To quantify the amount of HGF produced by HMF, enzyme-linked immunosorbent assay (ELISA) was performed with conditioned media collected from various HMF cultures. As for the previous gene expression experiments, we prepared low ($3x10^4$cells/well) and high ($6x10^4$cells/well) density 2D conditions, and 0.8mg/ml and 1.6mg/ml mixed matrix 3D conditions. In addition, we included pure collagen I matrices of the same concentrations to explore possible influences from soluble factors in Matrigel. We also included blank gels without any cells for both collagen I and mixed matrices as matrix controls. Media were collected at 24h and 48h. Almost twice as much HGF was detected as the collection time was doubled from 24 to 48 hours (data not shown). As can be seen in Fig. 4, HMFs in 3D conditions produced about twenty times more HGF than in 2D conditions after 48h culture. Similar results were obtained in both pure collagen I and the mixed matrix, suggesting no significant influence from the different ECM composition and the undefined soluble factors in Matrigel. Two blank gel controls and MCF-DCIS produced non-detectable amounts of soluble HGF. Along with the RT-qPCR data shown

previously, these data clearly suggest that HGF is secreted more in a 3D condition, further facilitating the invasive transition of MCF-DCIS cells.

Next, we examined the effect of HGF on the MCF-DCIS transition by inhibiting the transition through the neutralization of the HGF activity. We also developed a microfluidic *in vitro* co-culture system to further examine the effects of 2D and 3D culture conditions of HMFs on the MCF-DCIS transition. HGF neutralizing antibody binds to HGF and neutralizes any biological effect of HGF. Thus, 0.5 μg/ml of HGF neutralizing antibody was first added to conditioned media collected from 3D HMF culture and a transwell invasion assay was performed. As can be seen in Fig. 5, the neutralizing antibody added to the 3D conditioned media significantly reduced the number of invaded cells, and there was no difference from the conditioned media collected from a blank mixed matrix with and without the HGF neutralizing antibody. HGF is known to act through its cognate receptor, c-Met, which contributes to the growth and progression of a variety of solid human tumors (22; 25). Accordingly, we examined both HGF neutralizing antibody and c-Met inhibitor in a 3D microfluidic co-culture system, shown in Fig. 6A., to investigate if the blocking of HGF, and c-Met reduces the invasive transition of MCF-DCIS cells in an *in vitro* culture. We designed and optimized the system to enable combined co-culture of MCF-DCIS cells in a 3D condition together with HMF cells in either 2D or 3D conditions. The microfluidic co-culture system was composed of three connected cell-culture chambers. In the central chamber MCF-DCIS cells were placed in a 3D extra-cellular matrix, and in the two side chambers HMF cells were cultured in a 3D matrix or a 2D matrix. The central chamber was designed with a lowered height allowing the pinning of fluid in that region (26), such that the fluid can be flowed into the central chamber from either side chamber, but allowing the fluid to remain in the central chamber when aspirating fluid from the side chambers (Fig. 6B. and supplementary movie 1). The surface area and volume of the central chamber are 12.5mm^2 and 1.25μl respectively, and those of the two side chambers in total are 11.5mm^2 and 2.8μl respectively. The sample loading was completed in 3 simple steps (i.e., first injection, aspiration, and second injection), and it did not require the use of fluids with matching viscosities as other laminar flow patterning based devices do (15). After 6 days of cultivation, samples were fixed

and the morphology of MCF-DCIS clusters was analyzed. The addition of a HGF neutralizing antibody or a c-Met inhibitor to the 3D HMF/3D MCF-DCIS co-culture significantly decreased the invasive transition of MCF-DCIS cells as quantified by the decreased AR value shown in Fig. 6C and 6D. However, the morphology change was negligible when the antibody and inhibitor were added to the 2D HMF/3D MCF-DCIS co-culture. This is likely because the HMF cultured in the 2D condition did not originally produce a high enough amount of HGF, thus showing a negligible inhibition effect by the antibody and inhibitor.

Conclusions

The transition from DCIS to IDC is a critical stage in breast cancer progression, and improved understanding of the signaling mechanisms that regulate this transition can have clinical impact by identifying potential targets for alternative treatment options. But screening approaches using current 3D culture systems are cost prohibitive. To more efficiently investigate the signaling mechanisms in 3D, we need to establish and utilize a reliable *in vitro* system that closely recapitulates the DCIS microenvironment *in vivo*. In this study, we first presented the distinct differences between 2D and 3D *in vitro* cultures of HMF in secreting signaling molecules and how the differences influence the invasive transition of MCF-DCIS cells. While the importance of the 3D *in vitro* system and the influence of stromal fibroblasts in DCIS progression has been previously reported, this work clearly provides evidence that the 3D environment itself stimulated stromal fibroblasts and consequently caused increased secretion of signaling molecules. This data also brings empirical advantages. 3D cultures of HMFs increased the concentration of several molecules secreted from HMFs which in turn increased DCIS cell invasiveness without the use of engineered overexpressing cells as was used in previous studies. No difference in HGF expression was observed with different matrix compositions nor was HGF expression affected by blocking the function of $\beta 1$ integrin (Supplementary Fig. 2) suggesting a structural/mechanical mechanism may be involved in upregulating HGF in 3D culture conditions.

Further, we utilized a microfluidic *in vitro* system to provide a more efficient platform for the investigation of complex mechanisms involved in the cell-3D environment interaction. The microfluidic system enabled combined 2D/3D co-culture of MCF-DCIS and HMF cells using a simple pipette-driven loading process. Moreover, the side-by-side co-culture improved imaging capabilities by minimizing interference from the other cell type. The small volume required per endpoint and the compatibility with existing highthroughput infrastructure opens the door to the use of various neutralizing antibodies and small molecule inhibitors with minimal cost and labor enabling screening approaches in 3D culture.

Acknowledgements

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Materials and Methods

Cell culture

Human mammary fibroblast (HMF) cells were provided by Dr. Kuperwasser (27) and were cultured in DMEM with high glucose and L-glutamine (Invitrogen, 11965-092, Grand Island, NY) supplemented with 10% calf serum (Invitrogen, 26010074), and penicillin/streptomycin. MCF10-DCIS.com cells (28) were purchased from Asterand (Detroit, MI), and were cultured in DMEM-F12 with L-glutamine (Invitrogen, 11965-092) supplemented with 5% horse serum (Invitrogen, 11320-033), and penicillin/streptomycin. All cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO2.

Cell line authentication

MCF10DCIS.com cells were authenticated by using "Cell Check" service provided by RADIL(http://www.radil.missouri.edu) on the date of September 26, 2011. The sample was confirmed to be of human origin and no mammalian inter-species contamination was detected. The alleles for 9 different markers were determined and the results were compared to the alleles reported for a previously submitted sample from Asterand. The genetic profile for the our sample was identical to the genetic profile of the Asterand sample reported previously.

Microchannel design, fabrication, and operation

The microfluidic devices were fabricated using multilayered SU-8 molds and PDMS-based soft-lithography. In brief, three layers of SU8-100 (Microchem Corp), of thicknesses 100 µm, 150 µm, and 500 µm, were spun on a 150 mm diameter silicon wafer and patterned according to the manufacturer's guidelines. UV lithography was performed using an Omnicure 1000 light source (EXFO) using masks printed on transparency (ImageSetter, Madison, USA). Subsequently, the wafer was developed using SU-8 developer (PGMEA, Sigma) and cleaned in Acetone and IPA. Polydimethylsiloxane (Sylgard 184, Dow Corning) was mixed in a 1:10 cross-linker to base ratio, degassed for 30 min, and poured over the clean wafer on a hot plate. The molding process was performed by layering a transparency film, a layer of silicone foam, a 75 mm by 100 mm slab of glass, and a 5 kg weight on top of the wafer and PDMS, and baking the stack at 80 degrees C for 3 hours. The cured PDMS layers were peeled off of the wafer, sterilized in 70% ethanol, and attached to polystyrene cell culture dishes (TPP AG, Switzerland). For multiphoton and confocal laser scanning microscopy, PDMS channels were attached to a glass bottom culture dish (P50G-0-30-F, MatTek corp, Ashland, MA) after treating both the PDMS layer and the petridish in a plasma chamber for 50 seconds at 100W.

For loading, a cell suspension containing MCF-DCIS cells and a mixed matrix was inserted in one of the input ports until the fluid filled the center circular chamber. The excess cells in the side channels was removed by applying a gentle vacuum to the loading port. The cells-in-gel suspension was polymerized in a cell-culture incubator for 10 minutes by manually flipping the channels upside down every 2 minutes to prevent excessive cell settling. Subsequently, the two

side chambers were loaded with either a cell suspension of HMF cells in media, or in a mixed matrix.

Sample preparation for *in vitro* 3D culture

Collagen was prepared initially at a concentration of 5.0 mg/ml by neutralizing an acidic collagen solution (Collagen I, 354249, BD Biosciences) with 100mM HEPES buffer in 2X PBS (pH 7.7). For the collagen I only matrix condition, cells and culture media were added to neutralized collagen I gel to achieve a final concentration of 1.6 mg/ml. For mixed gel conditions, neutralized collagen gel and Matrigel (Basement Membrane Matrix, 356231, BD Biosciences) were mixed with equal volume, and the collagen I concentration (0.8 mg/ml and 2.0 mg/ml) was adjusted by cell suspension and culture media. For loading into microfluidic channels, the neutralized sample was kept at 4 °C for at least 15 min to apply an additional nucleation phase of collagen polymerization before channel loading (29).

Conditioned media collection

HMFs in a 2D condition proliferate faster than HMFs in a 3D condition, and, accordingly, we prepared lower cell densities for 2D samples ($3x10^4$ cells/48-well and $6x10^4$ cells/48-well) than the density of 3D samples ($1.2x10^5$ cells/48-well) to obtain similar final cell densities in the 2D and 3D samples after 48 hours. After cells are completely adhered to culture plates (for 2D samples) and to ECM (for 3D samples), serum-free media were added on top of samples. After 24 and 48 hours, conditioned media were collected and were centrifuged at 4000rpm for 5 minutes to pellet any floating cells and debris.

Invasion assay

The invasiveness of MCF-DCIS cells was assayed by transwell invasion chambers (Matrigel Invasion Chambers in two 24-well plates, 8.0 μm, 354480, BD Biosciences). We resuspended MCF-DCIS cells in serum-free DMEM/F12 ($5x10^4$cells/ml), and seeded in the upper compartment of chamber (0.2ml per chamber). The lower compartment was filled with 0.75ml of DMEM/F12 supplemented with different conditioned media collected from 2D and 3D cultures

of HMF as chemoattractant. After incubation at 37 °C in a humid atmosphere for 36 hrs, filters were rinsed with PBS. Remaining cells on the upper surface were wiped away with a wet cotton swab, and those on the lower surface were fixed with 4% paraformaldehyde, and stained with Hoechst (Hoechst 33342, H3570, Molecular Probe). The number of invaded cells per microscopic view was counted and averaged.

Proliferation assays

For proliferation assays, 2D and 3D samples were fixed at each time point (0, 24hr, and 48hr) and stained for nuclei with ToPro3. Cells were washed with 1xPBS then fixed with 4% paraformaldehyde for 30 min, and permeablized with 0.1% Triton X-100 in 1xPBS for 30 min at room temperature. ToPro3 was diluted 1:500 in PBS and incubated for 4 hours at room temperature, then washed three times with 1xPBS. The number of cells were estimated by scanning samples on an infrared (IR) laser scanner (Odyssey Licor Biosciences) to quantify integrated infrared intensity of ToPro3. The IR signal was calibrated by quantifying intensity values from different cell densities for 2D and 3D samples prior to perform proliferation assay (Supplementary Fig. 1).

Immunofluorescent staining

The samples were fixed in 4% paraformaldehyde in 1xPBS for 30 min at room temperature and, after 3 times washing with 1xPBS, the cells were permeablized with 0.1% Triton X-100 in 1xPBS for 30 min at room temperature. For filamentous actin staining, phalloidin solution (1:50, alexa fluor 594 phalloidin, Invitrogen) was added, incubated at 4 °C for overnight, and washed 3 times with PBS.

Imaging and analysis

Brightfield images were acquired on an inverted microscope (Eclipse Ti-U, Nikon) using the NIS-Element imaging system (Diagnostic Instruments, Inc.). F-actin and collagen fibers were imaged by using multiphoton laser scanning microscopy (with second harmonic filter for collagen). All Multiphoton laser scanning microscopy (MPLSM) and Second Harmonic

Generation (SHG) imaging was done on an optical workstation that was constructed around a Nikon Eclipse TE300. A MaiTai Deepsee Ti:sapphire laser (Spectra Physics, Mountain View, CA) excitation source tuned to 890 nm was utilized to generate both Multiphoton excitation and SHG. The beam was focused onto the sample with a Nikon (Mehlville, NY) 20X Super Fluor air-immersion lens (numerical aperture (NA) = 1.2). All SHG imaging was detected from the back-scattered SHG signal with a H7422 GaAsP photomultiplier detector (Hamamatsu, Bridgewater, NJ), and the presence of collagen confirmed by filtering the emission signal with a 445 nm (narrow-band pass) filter (TFI Technologies, Greenfield, MA) that isolated the SHG signal. Acquisition was performed with WiscScan (http://www.loci.wisc.edu/software/wiscscan), a laser scanning software acquisition package developed at the Laboratory for Optical and Computational Instrumentation. The morphology analysis of MCF-DCIS clusters was done by using shape descriptor measurement of ImageJ software for aspect ratio (major axis over minor axis).

mRNA Transcription Analysis

mRNA was isolated from 2D or 3D cultured cells in 24-well using Dynabeads® mRNATM DIRECT kit (Invitrogen, Cat# 610.21). Then mRNA was reverse transcribed to cDNA using high capacity cDNA reverse transcription kits from Applied Biosystems (Cat# 4374966). Real-time PCR was performed on StepOne Real-Time PCR System (Applied Biosystem) using TaqMan qPCR master mix (Applied Biosystems) along primer/probe sets from Applied Biosystems for the HGF (Hs00300159_m1), MMP14 (Hs01037009_g1), COX2 (Hs01573471_m1), CXCL12 (Hs00171022_m1), and GAPDH (Hs99999905_m1) used as a housekeeping gene to normalize the total number of molecules in each sample. All PCR products had a denaturing step of 95 °C for 15 s, an annealing/extension step at 60 °C for 1 min for a total of 40 cycles. Quantification of mRNA was calculated using relative standard method. Standards are composed of five 1:10 serial dilution of the same gene with higher amount of starting cDNA.

Zymography of MMPs Activity

To determine gelatinolytic and caseinolytic activities in HMF conditioned medium, zymography was performed using gelatin and casein zymogram gels (Invitrogen). Conditioned medium from 2D and 3D culture of HMF cells was collected at 48 hours culture. After being clarified by centrifuge, samples were mixed with 2xSDS sample buffer (Invitrigen) and then subject to electrophoresis separation at 100V for 90 minutes. The gels were soaked in Renature buffer for 30min at RT and equilibrated in Develop buffer for 30 minutes. Then gels were incubated with Develop buffer overnight at 37°C to allow proteinase digest of its substrate. Gels were stained using GelCodeTM Blue stain reagent (PIERCE) for two hours and then distained by DI water. Proteolytic activities appeared as clear bands of lysis against a blue background of stained gelatin or casein. To verify that the detected gelatinolytic and caseinolytic activities were specifically derived from MMPs, the gels were treated in parallel experiments with develop buffer containing 20mM of EDTA.

HGF ELISA

Conditioned medium from 2D and 3D culture of HMF cells was collected and clarified as above. HGF ELISA kit (Invitrogen) was used to determine HGF in conditioned medium. Briefly, 50 µl standard dilutions of recombinant human HGF and experimental conditioned media were dispensed into a 96-well plate coated with anti-HGF. The plate was sealed, incubated at room temperature for 3 hours and washed four times with washing buffer. After addition of 100 µl of biotinylated anti-Hu HGF solution and incubation for 1 hour at RT followed by four washes, 100 µl of Streptavidin-HRP was added and incubated for 30 minutes at RT. After 4 times of wash, 100µl of Stabilized Chromogen was added to the wells and incubated for 30 minutes, followed by addition of 100 µl of Stop solution. Read the absorbance of each well at 450 nm using SpectraMax Plus Spectrophotometer.

Figure captions

Fig. 1. 3D *in vitro* culture of HMF induce an increased transition of MCF-DCIS cells. (A) Representative image of MCF-DCIS clusters showing side branches. F-actin is visualized by

alexa 594 phalloidin (red) and collagen 1 structure is visualized by second harmonic generation (green). Scale bar is 30 μm. (B) Outlines of MCF-DCIS clusters cultured in 3D mixed matrix with conditioned media collected from 3D HMF culture and 2D HMF culture. The clusters cultured with conditioned media from 3D HMF culture produced more elongated clusters with aspect ratio (AR) 1.57. Scale bar is 100 μm. (C) Bar graph showing average aspect ratio of MCF-DCIS clusters cultured with control (serum free media), 2D CM (conditioned media from 2D HMF culture), and 3D CM (conditioned media from 3D HMF culture). * represents p value of 0.048. (D) Bar graph showing data obtained from transwell invasion assays with 2D CM and 3D CM.

Fig. 2. Gene expression analysis of stromal derived signaling molecules. (A) a table showing loading conditions for 2D and 3D samples. (B) Proliferation curves for HMFs cultured in 2D (blue) and 3D (green) conditions. After 48 hours of culturing, the cell densities of HMFs in 2D and 3D became similar (P=0.26). (C) Bar graphs showing the mRNA expressions of HGF, MMP-14, COX2, and CXCL12 at each loading condition. (D) Zymography showing the presence of increased active MMP-2 in the 3D CM.

Fig. 3. HGF mRNA expressions in MCF-DCIS cells cultured in 2D and 3D conditions.
HGF mRNA was undetectable in MCF-DCIS cells in both 2D and 3D culture conditions. HGF mRNA expression in HMF cells was used as a positive control.

Fig. 4. Increased secretion of HGF from HMF cells in 3D culture. (A) Conditioned medium from HMF cells cultured either on 2D surface or in 3D matrix (Collagen I or Matrigel and Collagen mixed gel) for 48 h were collected. HGF in conditioned media were analyzed by ELISA. HMF cells in 3D cultures significantly increased the secretion of HGF. The relative increase of HGF in conditioned media in 3D verse 2D is 20 folds.

Fig. 5. Invasion of MCF-DCIS cells with HGF neutralizing antibody (anti HGF) using transwells. The HGF neutralizing antibody (0.5 ug/ml) is added to 3D CM and BK CM (conditioned media collected from blank mixed gels). * represents p value of 0.034.

Fig. 6. (A) Design of microchannels used for 2D and 3D combined co-culture of HMF and MCF-DCIS cells. The illustration for loading process shows two simple pipette injections and one aspiration. (B) Demonstration of channel loading using red and blue food coloring dyes. Scale bar is 5 mm. (C) MCF-DCIS clusters (red and outline) and collagen architecture (green) co-cultured with 3D HMF (3D/3D) and with 2D HMF (2D/3D). Higher SHG signal (Green) shows higher collagen density and more architecture alteration. The bottom four images show MCF-DCIS clusters co-cultured with the addition of neutralizing HGF antibody at 0.5 um/ml (anti-HGF). Scale bar is 100 μm. (D) Bar graphs showing MCF-DCIS cluster shape analysis by estimating averaged aspect ratio. Both HGF neutralizing antibody and c-met inhibitor (anti c-met) decreased the aspect ratio of MCF-DCIS clusters in 3D/3D co-culture. * represents p value is less than 0.05.

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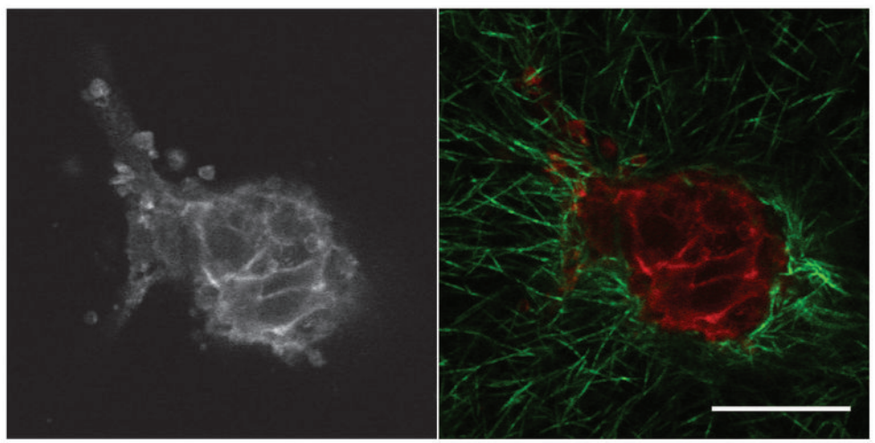
Figure 1

A



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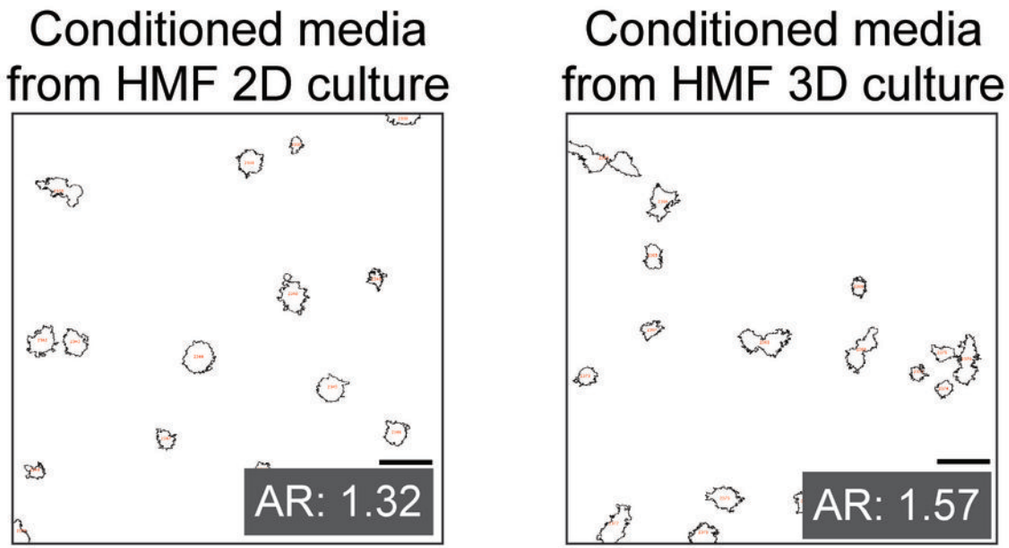
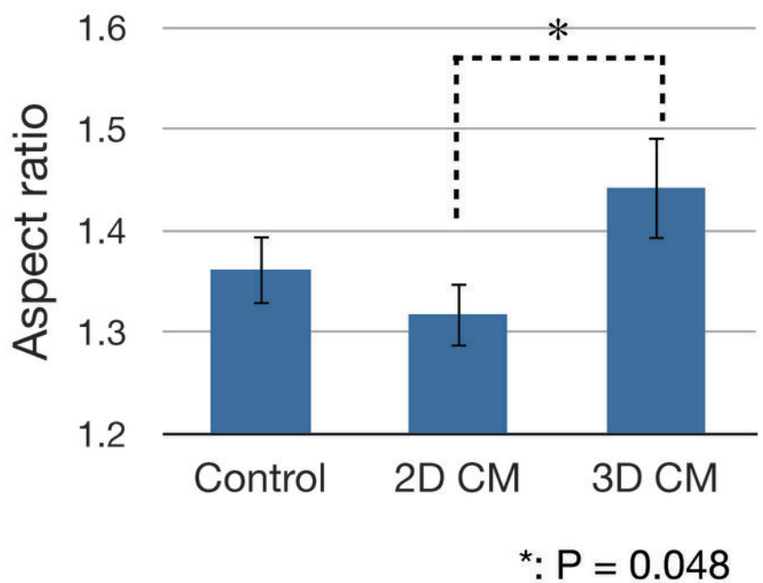


C



D

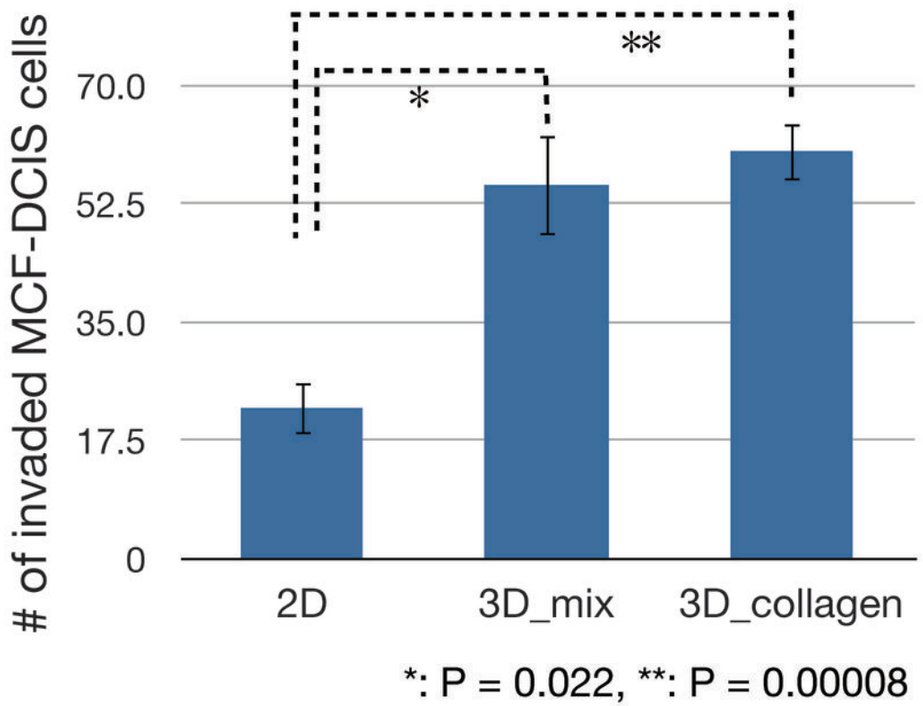
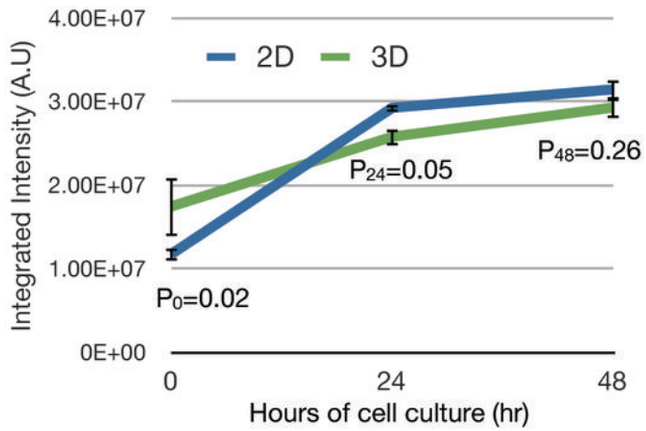


Figure 2

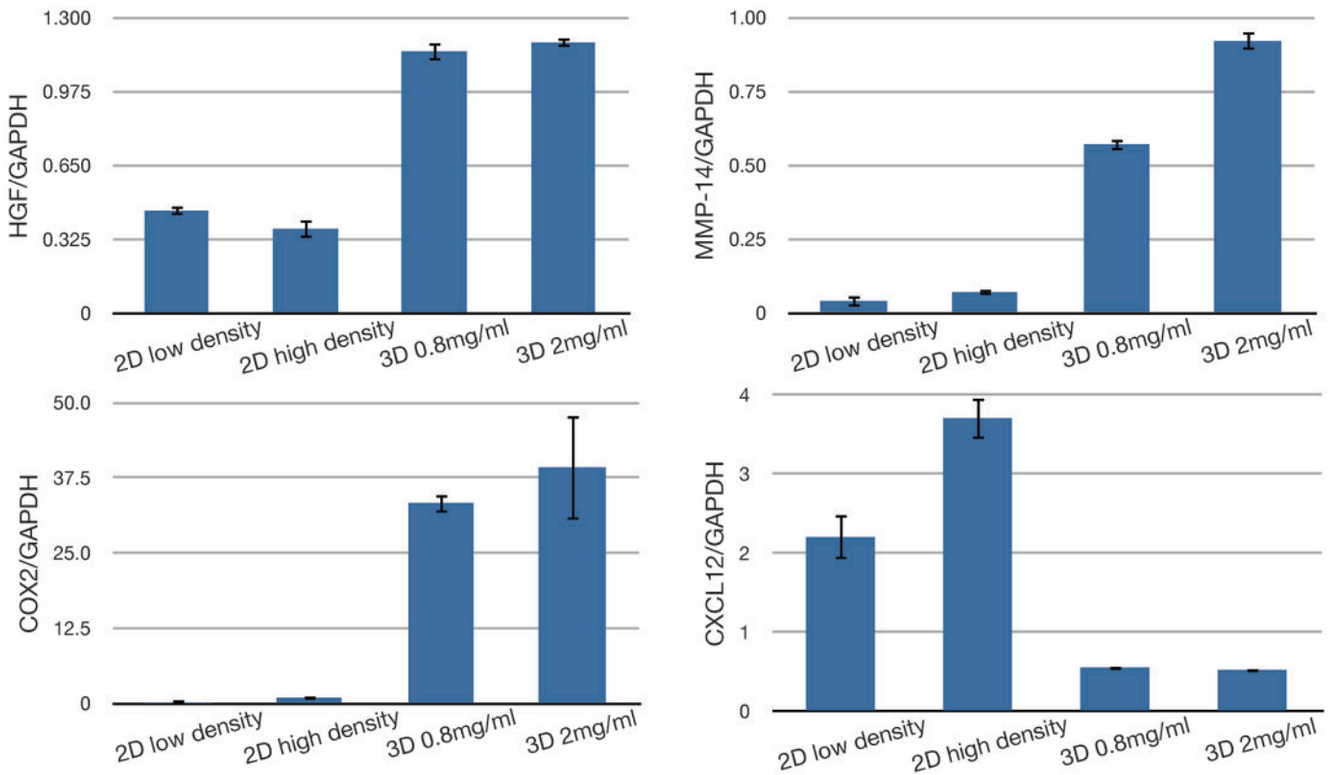
A

Loading conditions	Cell loading density [E+05cells/48-well]	Collagen I conc. [mg/ml]
2D_low	0.3	0
2D_high	0.6	0
3D_0.8	1.2	0.8
3D_2.0	1.2	2

B



C



D

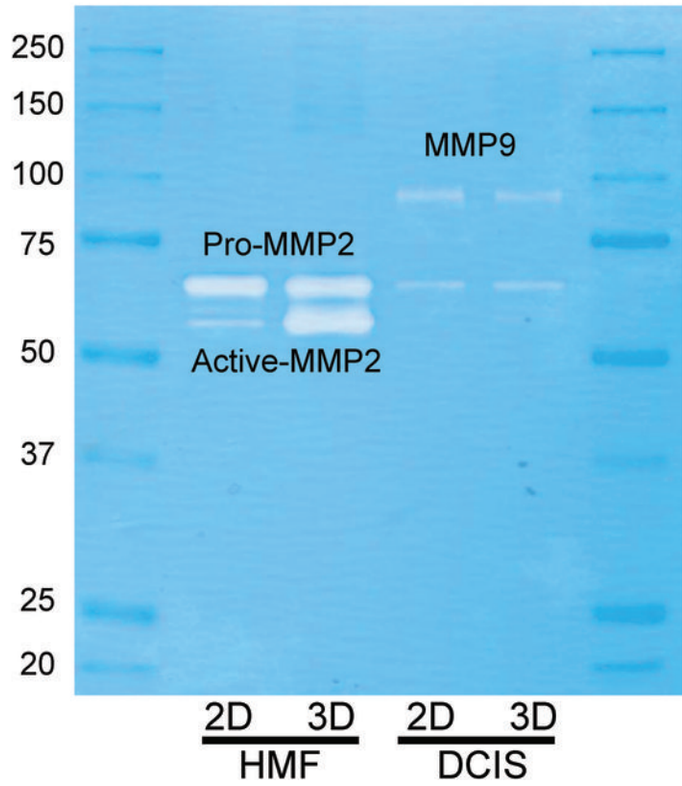


Figure 3

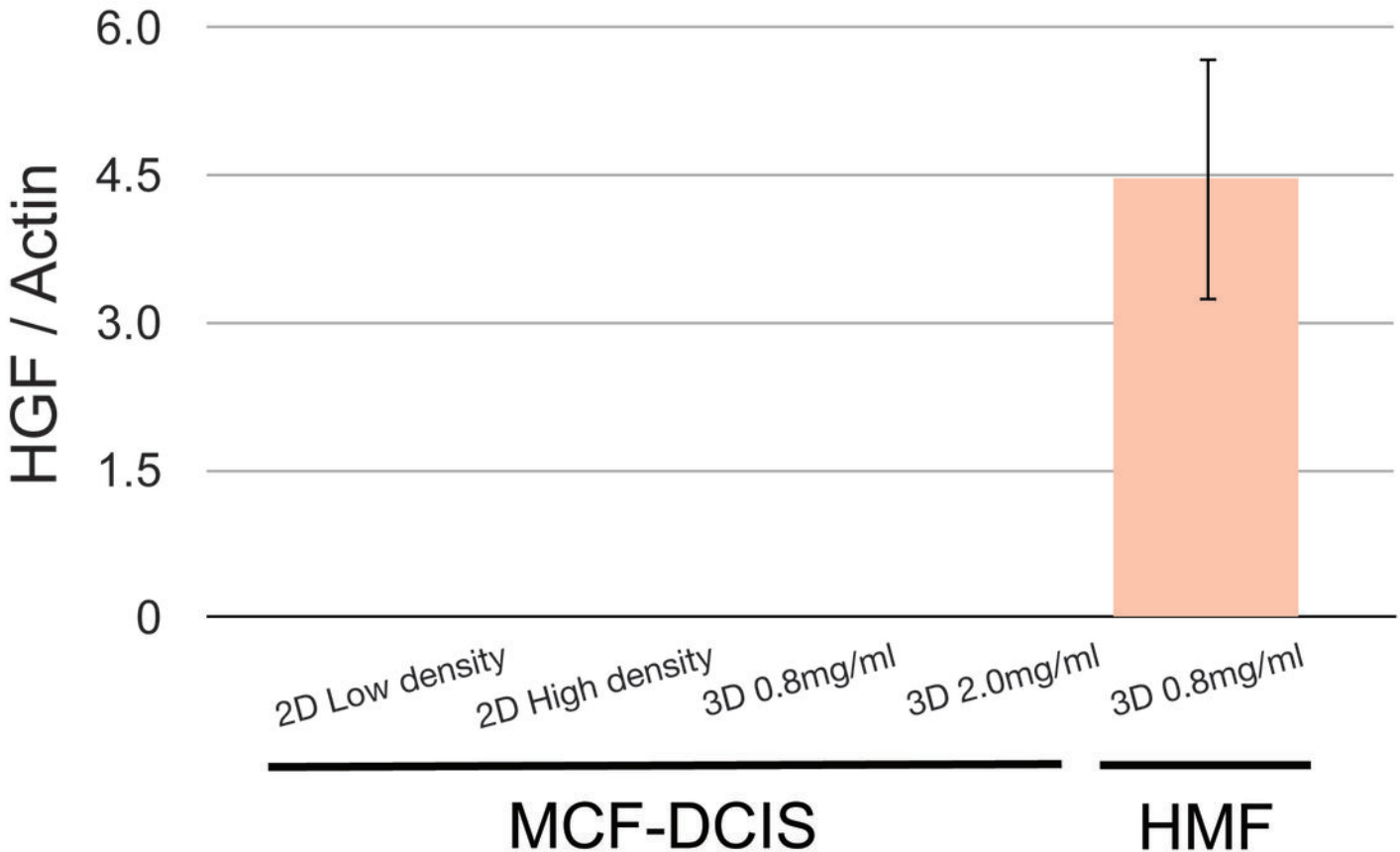

6.0
4.5
3.0
1.5
0
HGF / Actin
2D Low density
2D High density
3D 0.8mg/ml
3D 2.0mg/ml
3D 0.8mg/ml
MCF-DCIS
HMF

Figure 4

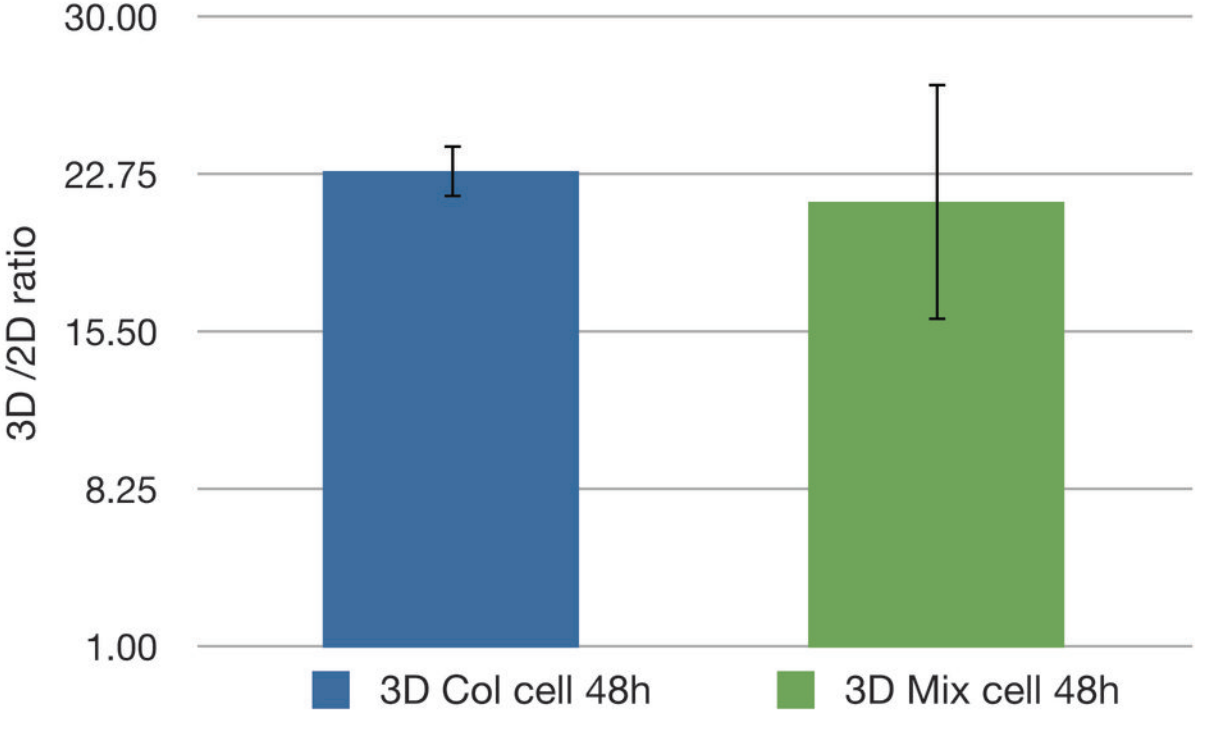
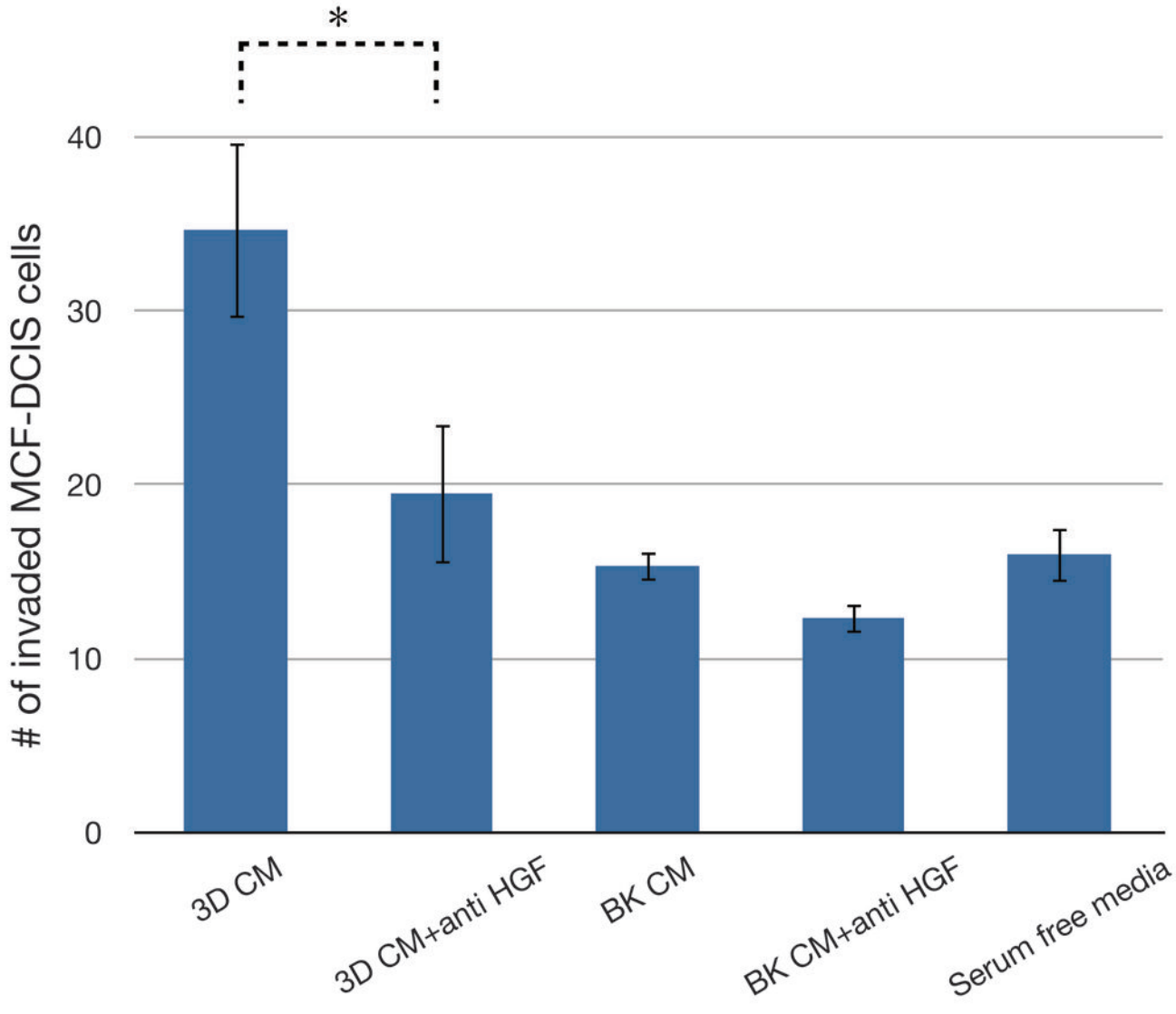
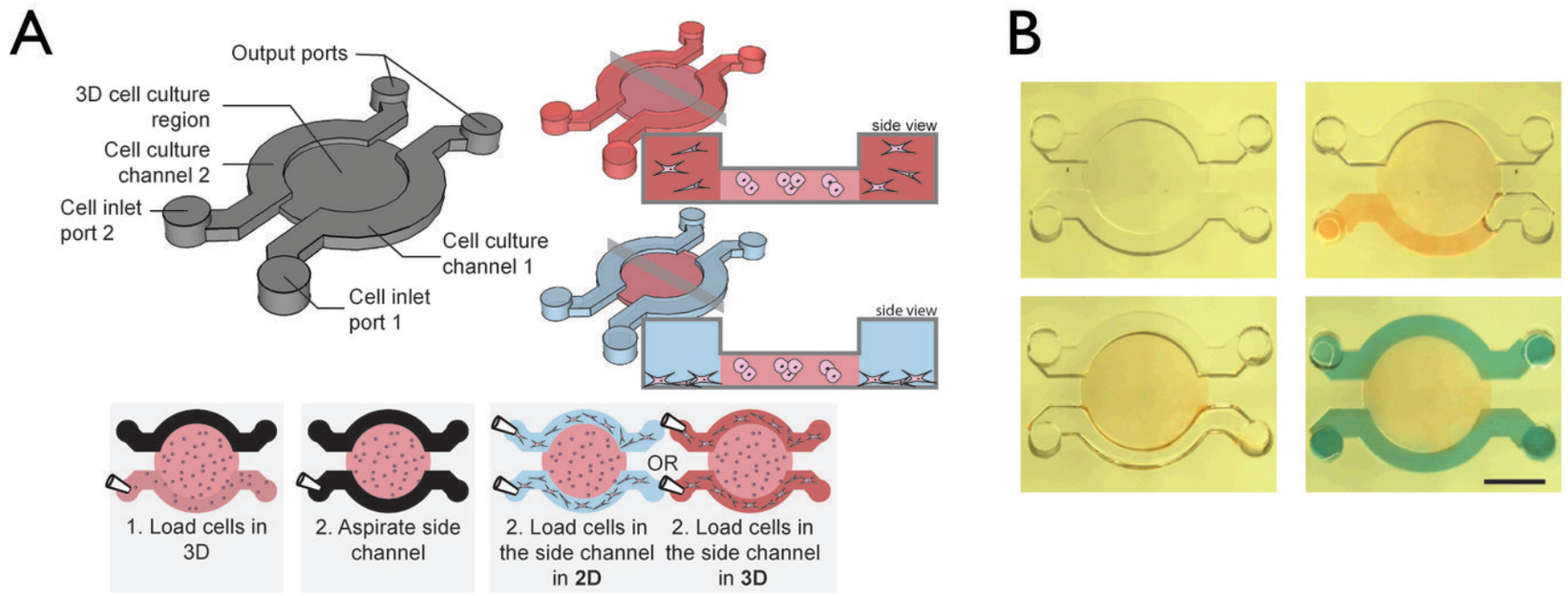

30.00
22.75
15.50
8.25
1.00
3D /2D ratio
3D Col cell 48h
3D Mix cell 48h

Figure 5

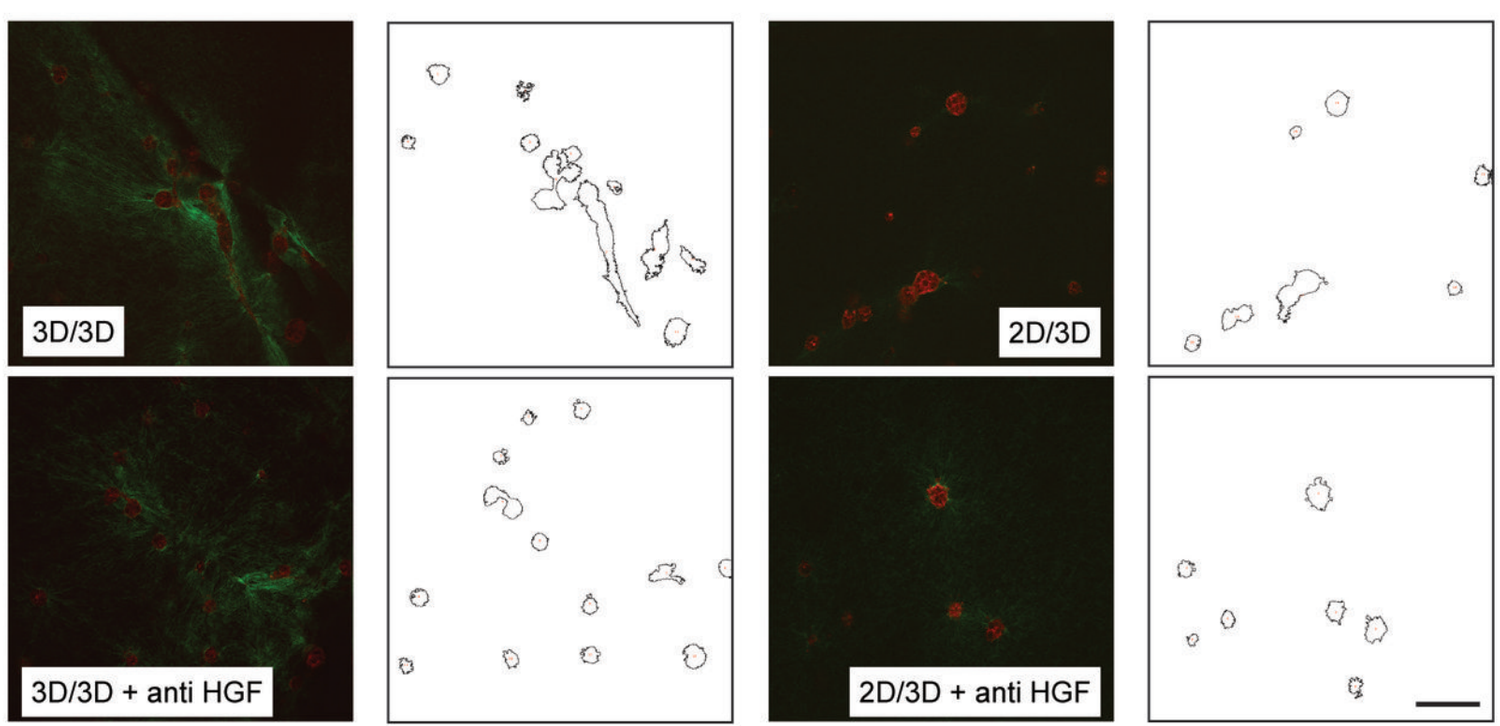


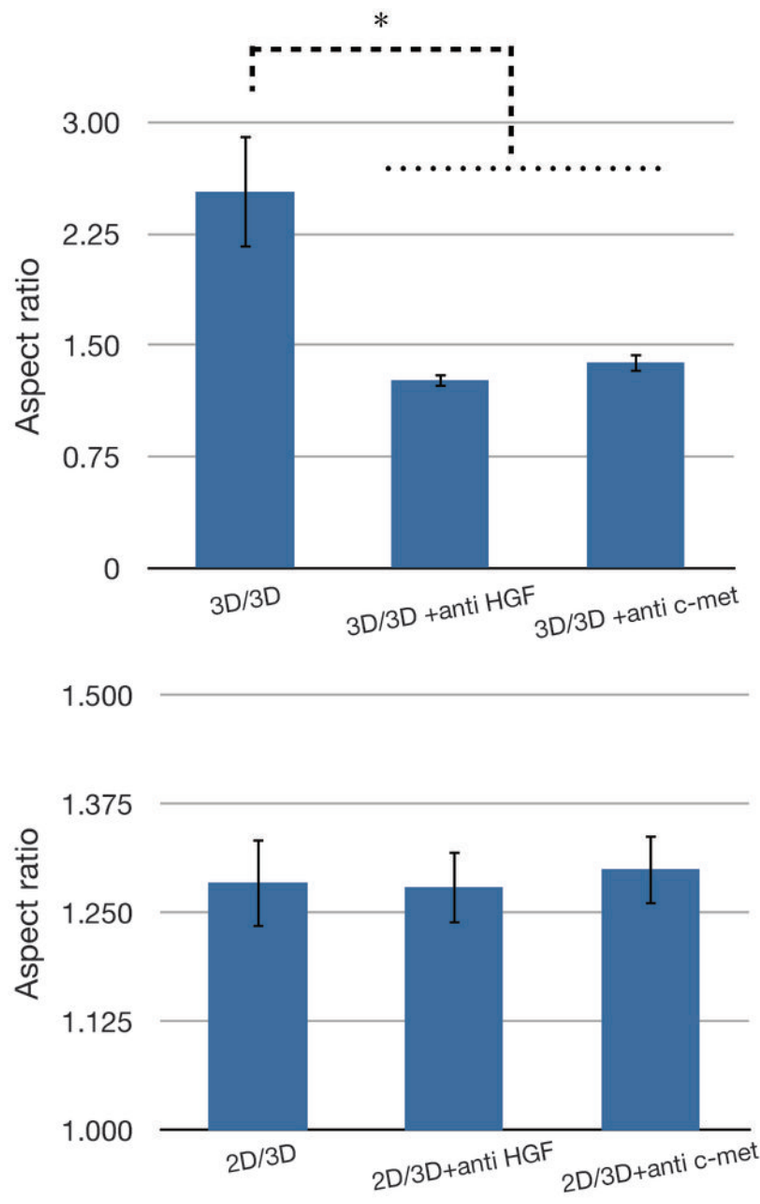
*: P = 0.034

Figure 6


A
Output ports
3D cell culture region
Cell culture channel 2
Cell inlet port 2
Cell culture channel 1
Cell inlet port 1
side view
side view
1. Load cells in 3D
2. Aspirate side channel
OR
2. Load cells in the side channel in 2D
2. Load cells in the side channel in 3D

B


C
3D/3D
2D/3D
3D/3D + anti HGF
2D/3D + anti HGF


D
*
Aspect ratio
3.00
2.25
1.50
0.75
0
3D/3D
3D/3D +anti HGF
3D/3D +anti c-met
Aspect ratio
1.500
1.375
1.250
1.125
1.000
2D/3D
2D/3D+anti HGF
2D/3D+anti c-met

Supplementary information

Blocking of beta 1 integrin does affect HGF secretion by HMFs in a 3D condition.

After we confirmed the different activities of HMFs cultured in 2D and 3D conditions, we hypothesized that the increased engagement of integrins such as the β1 integrin in a 3D condition is responsible for the increased secretion of HGF from HMF 3D culture. One of the major differences observed between 2D vs. 3D cultures is the physical adhesions to the surrounding ECM structures. The cell-matrix adhesion in 3D cultures is accompanied by the involvement of various integrins. The study by Fischbach et al. also indicated that the engagement of $\alpha 5\beta 1$ integrin in 3D microenvironment regulates cancer cell angiogenic capability (10). Integrins are trans-membrane adhesive receptors that are composed of heterodimeric subunits designated as alpha and beta and the integrins mainly link the ECM to the cytoskeleton. Among twenty-four heterodimeric subunits, the β1 integrin is part of most collagen and laminin receptors (30), and thus selected here to further investigate. Accordingly, we added an anti-β1 integrin antibody (25μg/ml) to a 3D culture of HMF to block the function of the β1 integrin and collected conditioned media after 48 hours for HGF ELISA assays. The HGF ELISA data showed no significant differences with or without the β1 integrin blocking antibody in 2D and 3D cultures (Fig. S2.), but the data did still present significant differences between 2D and 3D cultures of HMF. This result suggests that β1 integrin activation itself may not strongly contribute to the production of HGF. Based on the fact that there are 17 α subunits and 8 β subunits of integrins and these α and β subunits heterodimerize to produce 22 different complexes (31), it was not surprising to find that blocking of one specific integrin is not sufficient to disturb complex interactions between HMFs and various ECM compositions in the mixed matrix used in this work. Alternatively, integrins may play no role in regulating the secretion of HGF. Further investigations with other integrins or integrin complexes may provide better clues to

understanding the enhanced secretion of HGF from 3D cultures of HMFs and might also better reveal mechanisms involved in these phenomena.

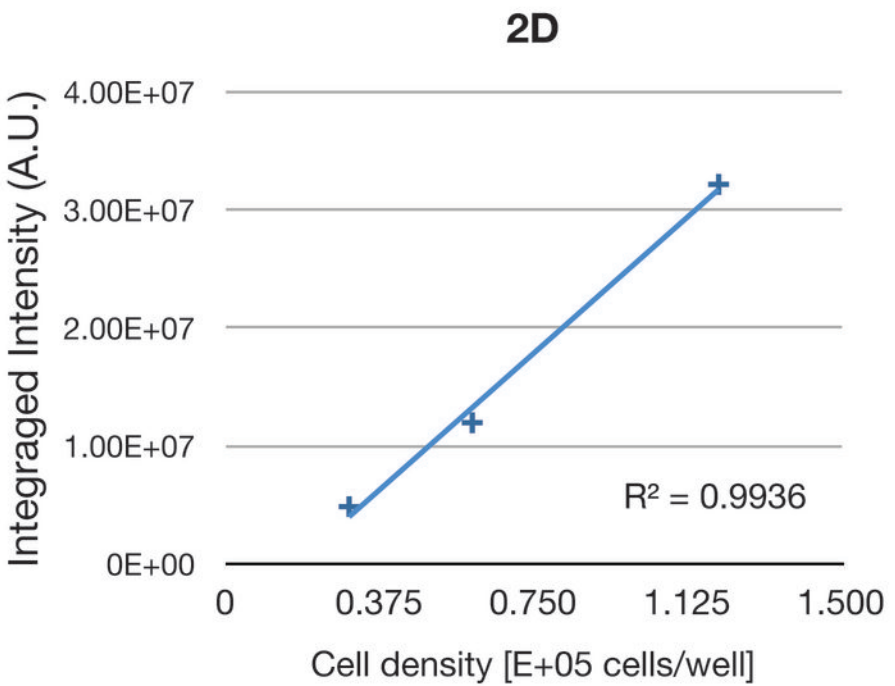
Supplementary figure captions

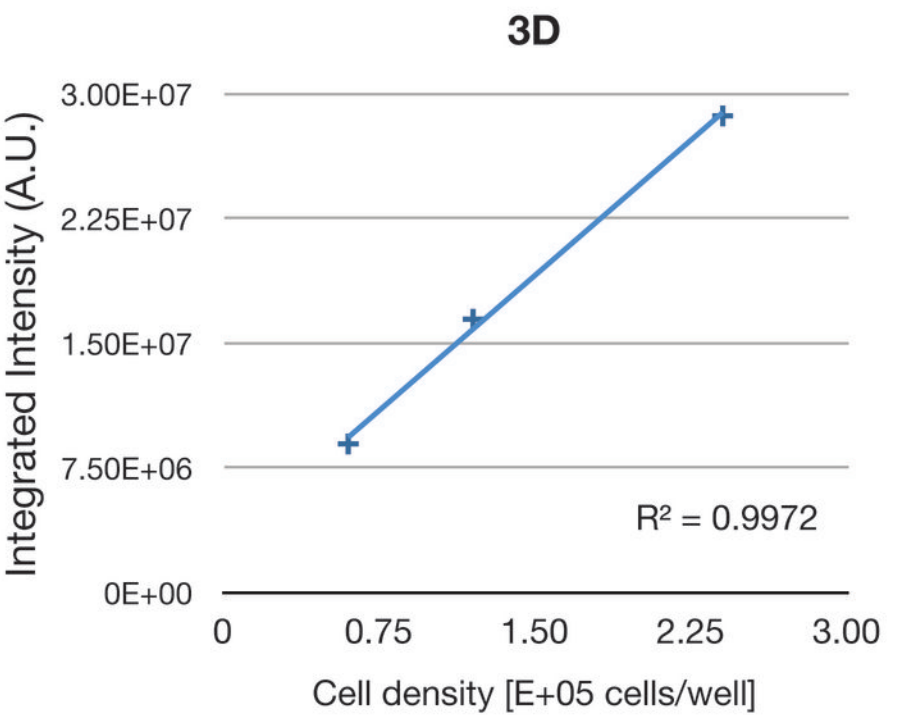
Fig. S1. Standard curve showing linear relationship between the number of cells in samples and the intensity of the infrared signal from the DNA-ToPro3 complex.

Fig. S2. The effect of β1 integrin function blocking antibody. Bar graph shows data from HGF ELISA performed with conditioned media collected from 2D and 3D cultures of HMF and also with the β1 integrin function blocking antibody (25ug/ml).

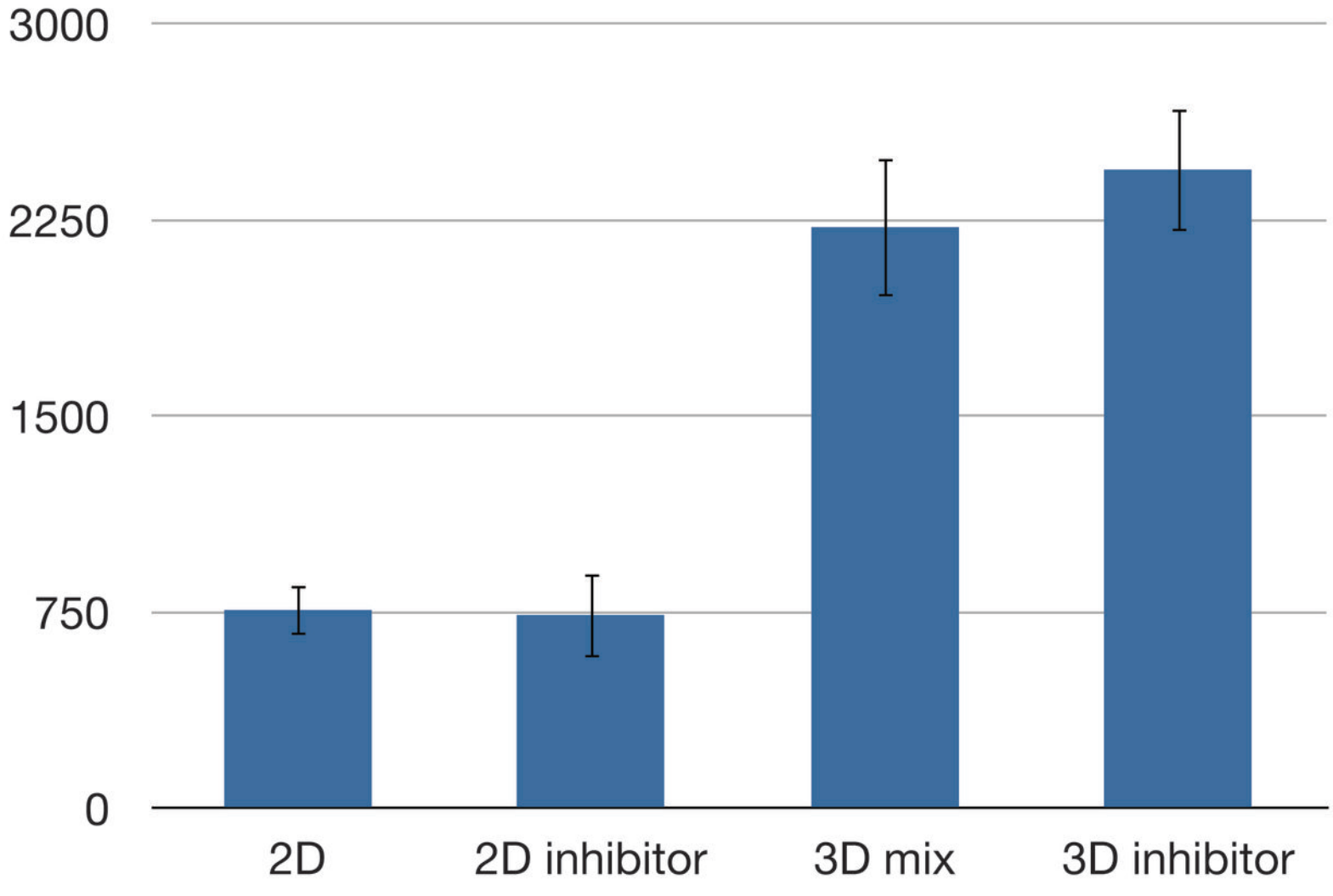
Movie. 1. A movie showing loading process into a three compartments microfluidic channel with red and blue food coloring dyes.

Supplementary Figure 1





Supplementary Figure 2



Journal of Biomolecular Screening

Enabling Screening in 3D Microenvironments: Probing the Matrix and Stromal Components Effect on the Morphology and Proliferation of T47D Breast Carcinoma Cells

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Keywords:	Automation or robotics, Cancer and cancer drugs, Cell-based assays, High content screening, Microfluidics
Abstract:	While the importance of the three-dimensional (3D) microenvironment and the extracellular matrix (ECM) composition in breast cancer is clear, current screening platforms are typically limited to two-dimensional (2D) cultures, and often exclude the influence of ECM and stromal components in modulating cellular behavior. Therefore, there is a need for screening platforms that culture cells in more in vivo-like 3D microenvironments rich in ECM proteins and stromal cells. In this work, an automated microfluidic platform was employed to screen for ECM compositions that affect the 3D morphology and proliferation of T47D breast carcinoma cells in monocultures and co-cultures with human mammary fibroblasts (HMF). First, 1.3mg/mL of collagen (CN) mixed with 0, 100µg/mL of fibronectin (FN), or 100µg/mL of laminin (LN) affected the circularity, aspect ratio, and cluster size of T47D cells. Second, CN mixed with 10µg/mL of FN or 10µg/mL of LN did not affect the growth of T47D monocultures. However, when co-cultured with HMF, the growth of T47D cells decreased. The platform presented in this work enables screening for the effects of matrix and stromal compositions and may facilitate the identification of new drug targets using more biologically relevant screening platforms.

Enabling Screening in 3D Microenvironments: Probing the Matrix and Stromal Components Effect on the Morphology and Proliferation of T47D Breast Carcinoma Cells

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Keywords: extracellular matrix, 3D, high throughput screening, breast cancer

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ABSTRACT

While the importance of the three-dimensional (3D) microenvironment and the extracellular matrix (ECM) composition in breast cancer is clear, current screening platforms are typically limited to two-dimensional (2D) cultures, and often exclude the influence of ECM and stromal components in modulating cellular behavior. Therefore, there is a need for screening platforms that culture cells in more in vivo-like 3D microenvironments rich in ECM proteins and stromal cells. In this work, an automated microfluidic platform was employed to screen for ECM compositions that affect the 3D morphology and proliferation of T47D breast carcinoma cells in monocultures and co-cultures with human mammary fibroblasts (HMF). First, 1.3mg/mL of collagen (CN) mixed with 0, 100μg/mL of fibronectin (FN), or 100μg/mL of laminin (LN) affected the circularity, aspect ratio, and cluster size of T47D cells. Second, CN mixed with 10μg/mL of FN or 10μg/mL of LN did not affect the growth of T47D monocultures. However, when co-cultured with HMF, the growth of T47D cells decreased. The platform presented in this work enables screening for the effects of matrix and stromal compositions and may facilitate the identification of new drug targets using more biologically relevant screening platforms.

INTRODUCTION

The mammary gland is a dynamic tissue in which cells in the mammary epithelium continuously interact with cells in the surrounding microenvironment. When the microenvironment receives signals from cells in the mammary epithelium, it sends back cues that help to maintain normal mammary tissue functions. If these interactions are disturbed, changes in the morphology, differentiation, proliferation, and migration of cells occur that can ultimately lead to the formation of a tumor and its progression to malignancy. It is believed that the major contributors to these changes are genetic alterations within the epithelial cells. However, evidence suggests that the extracellular matrix (ECM) composition also influences these interactions.

The ECM is composed of different molecules with specialized properties that not only provide a physico-mechanical and geometrical scaffolding to cells, but also influence cell behavior[1]. Some of the major ECM proteins found in the mammary gland include collagens, fibronectin (FN), and laminin (LN). Type-I collagen (CN) is the major fibrillar component in the mammary gland and serves as a backbone that provides structural integrity to the mammary gland, whereas FN and LN regulate cell adhesion to the ECM. Therefore, the interactions between these ECM components and mammary epithelial cells are important for maintaining normal mammary gland tissue functions. In fact, previous studies have shown that luminal epithelial cells polarize, resemble acini structures similar to those seen in vivo, and express milk proteins in response to lactogenic hormones when cultured in a three-dimensional (3D), LN-rich ECM gel[2]. However, if luminal epithelial cells are cultured using traditional, 2-dimensional (2D) surfaces or CN gels lacking LN, the cells lose their polarity and mammary-specific gene

expression. These results demonstrate that both the 3D microenvironment and the ECM composition play a critical role in guiding normal mammary tissue function.

While the importance of the 3D microenvironment and the ECM composition is clear, current ECM screening platforms are typically limited to the 2-dimensional (2D) culture of cells, and often exclude the influence of stromal cells in modulating cellular behavior. For example, cellular microarrays have previously been used as ECM screening platforms to study the effect of the ECM composition in the differentiation of human mammary progenitor cells[3] and the proliferation and activation of hepatic stellate cells[4]. Multiple soluble formulations have also been included within cellular microarrays to examine the effect of growth factors in the growth and differentiation of embryonic stem cells[5]. Although cellular microarrays provide a convenient screening platform to study cellular behavior under different ECM compositions, they are typically limited to the 2D culture of cells on top of ECM patterns and do not represent the 3D microenvironment that is observed in vivo. Additionally, media is typically shared across the array such that results are confounded by soluble factors cross talk between array locations. Moreover, little work has been done to develop cellular microarrays that include stromal cells as part of the ECM microenvironment. This is particularly important in breast cancer research since stromal fibroblasts have been shown to interact with carcinoma cells during cancer development by modulating carcinoma cell proliferation both in vivo[6] and in vitro[7]. Therefore, there is a need for more biologically relevant screening platforms that provide cancer cells with 3D microenvironments that are rich in ECM molecules and stromal cells, while providing independent experimental conditions.

In this work, an automated microfluidic platform previously developed for 3D cell culture[8] was employed to screen in 3D microenvironments of various ECM compositions that

affect the morphology and proliferation of T47D breast carcinoma cells in monocultures and in co-cultures with human mammary fibroblasts (HMF). Seven combinations of three different ECM molecules (CN, FN, LN) were used and the circularity, aspect ratio, and size of T47D clusters were screened to examine the effect of the ECM composition on the 3D morphology of T47D cells. The proliferation of T47D cells was examined under the influence of the different ECM compositions with and without stromal cells. Applying the concepts presented in this work to higher throughput screening platforms promises to be useful for studying cell-ECM interactions in more physiologically relevant 3D *in vitro* conditions and predicting the response of breast cancer cells *in vivo*.

MATERIALS AND METHODS

Cell culture and ECM gels preparation

Human T47D breast carcinoma cells were cultured in flasks with low-glucose DMEM (1.0mg/mL, Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY). Human mammary fibroblasts immortalized with telomerase (HMFs) were cultured in high glucose DMEM (4.5mg/mL, Gibco), supplemented with 10% calf serum (CS) and 1% penicillin/streptomycin. Both cell lines were cultured in separate flasks inside a humidified incubator at 37°C and 5% CO_2 before mixing with ECM gels and seeding in microchannels.

Extracellular matrix gels were prepared by mixing CN with FN or LN to get a total of seven different ECM compositions. FN (1mg/mL, human; BD Biosciences, Bedford, MA) and LN (1.88mg/mL, mouse; BD Biosciences) were reconstituted as specified by the manufacturer. A stock solution of CN (3.64 mg/mL, rat tail; BD Biosciences) was neutralized with a solution of 100mM HEPES buffer in 2X PBS in a 1:1 ratio, and incubated inside a bucket with ice for 10

minutes. Cells were resuspended in serum-free DMEM (SF-DMEM). FN or LN were mixed with the neutralized CN and final ECM concentrations were adjusted by adding SF-DMEM to obtain 1.3 mg/mL of CN with 0, 10, 50 or 100μg/mL of FN or LN and a final cell density of 6 x 10^5cells/mL (approximately 700cells/microchannel). In co-culture experiments, HMF and T47D cells were added to the ECM gels in a 1:2 (HMF: T47D) ratio.

Tubeless microfluidic device fabrication and automated loading

Polydimethylsiloxane (PDMS) tubeless microfluidic devices were fabricated as described previously[8]. The dimensions of the PDMS microchannels array (MCA) with straight microchannels (0.75 mm wide, 0.25 mm high, and 4.5 mm long) are shown in Figure 1.

The automated liquid handler used to load microchannels was optimized and described previously[8]. In this work, the same platform was used to culture T47D cells in different ECM compositions in the presence and absence of HMF cells. The ECM/cell mixtures were manually pipetted to seven wells of a 96-well plate and the automated platform was used to load microchannels as described previously[8]. Seven combinations of three different ECM proteins (CN, FN and LN) were used to culture T47D cells in monocultures (first 5 microchannels per row, Figure 1A) and in co-cultures with HMF cells (last 5 microchannels per row, Figure 1A). Moreover, the MCA included 5 replicates for each ECM/cell combination that resulted in a total of 70 microchannels. After the loading was done, the MCA was kept inside a 37°C incubator for seven days. Media changes were done every other day.

Immunofluorescent staining

For the quantification of T47D cluster size, cells in the MCA were stained using the automated platform as described previously[8]. T47D and HMF cells were stained with primary

antibodies against pan-cytokeratin (CK, 1:75 dilution ratio, mouse monoclonal antihuman pan-cytokeratin; LabVision, Fremont, CA) and vimentin (VM, 1:150 dilution ratio, rabbit polyclonal antihuman vimentin; LabVision, Fremont, CA). Secondary antibodies were added in a 1:150 dilution ratio (Alexa Fluor 594 goat antimouse; Alexa Fluor 488 goat antirabbit; Invitrogen, Carlsbad, CA). For counterstaining the nuclei, Hoechst 33342 was used at 20μg/mL (H3570; Invitrogen, Carlsbad, CA).

Image acquisition and analysis

Fluorescence imaging of T47D and HMF cells was performed on an inverted microscope (Eclipse Ti, Nikon Instruments, Melville, NY) using the NIS-Element imaging system (Diagnostic Instruments, Sterling Heights, MI). The high-throughput data analysis platform, JeXperiment (http://jexperiment.wikidot.com), was used to perform the microscopy image processing and data mining. The JeXperiment platform allowed importing data collected from each microchannel into a database, and managed the data processing for each microchannel with custom user algorithms or functions chosen from a library. Custom analysis algorithms were made to plug into the JeXperiment workflow and enabled the quantification of circularity, aspect ratio, cluster size, and total staining area of CK-positive clusters. Circularity was measured with the formula of 4π x area/perimeter^2. Aspect ratio was defined as the ratio of major axis over minor axis. A rolling-ball background-subtraction algorithm was applied to determine a threshold value to obtain binary masks (Figure 2). The ImageJ (Rasband, W.S., ImageJ; U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997-2009) particle analyzer was applied to obtain the circularity, aspect ratio and size of T47D clusters.

RESULTS AND DISCUSSION

FN and LN are two important ECM molecules that change their expression levels during breast cancer development. Changes in the expression of these proteins can affect the interactions between cells in the epithelium and the stroma that could contribute to the development of breast tumors. In this work, we hypothesized that the automated microfluidic platform presented before[8] would enable screening for the effects of the stoma and ECM composition on T47D breast carcinoma cells behavior. Figure 1A shows a representation of a 10 by 7 microchannel array (MCA) used to culture T47D cells in monoculture (first 5 channels in a row) and in co-culture with HMF cells (last 5 channels in a row). ECM compositions contained CN as the major ECM protein, and FN and LN were mixed with CN to obtain seven different ECM compositions (see Figure 1B). Previously, the 3D morphology [9,10] and proliferation [7, 11] of breast cancer cells were shown to be useful readouts to investigate breast cancer cell behavior. Therefore, in this work the 3D morphology and proliferation of T47D cells were used as endpoints to evaluate the influence of the stroma and ECM composition in breast cancer.

3D morphology of T47D clusters in different ECM compositions

Examining the 3D morphology of breast cancer cells can provide important information. For example, 3D microenvironments have been shown to affect the 3D morphology and gene expression patterns of different breast cancer cell lines[10]. Also, the rigidity of the microenvironment affected the morphology of T47D cells, resulting in the down-regulation of Rho and FAK function[12]. More recently, changes in the circularity and aspect ratio of MCFDCIS.com cells were used as primary readouts for studying the transition from ductal carcinoma in situ (DCIS) to the invasive ductal carcinoma (IDC)[9]. In this work, we hypothesized that changes in the ECM composition would affect the 3D morphology of T47D clusters. In

order to test this hypothesis, the morphology of T47D cells cultured in 3D microenvironments of different ECM compositions was examined in the presence and absence of HMF cells.

Figure 2 shows immunofluorescence (left panel) and binary mask (right panel) images of T47D cells loaded inside microchannels in monocultures and in co-cultures with different ECM compositions. In 1.3mg/mL collagen type-I gels (CN), T47D cells had smaller and more circular clusters in monocultures than in co-cultures with HMF cells. This agrees with previously reported data where T47D cluster size did not increase in monocultures during a seven days culture period, but increased significantly when co-cultured with HMF cells[11]. However, in collagen gels containing 100μg/mL of fibronectin (100FN) T47D clusters adopted a less circular and more elongated morphology in co-cultures when compared to co-cultures with only CN. Adding 100μg/mL of laminin to collagen type-I gels (100LN) did not affect the circularity of the clusters, but reduced the amount of small T47D clusters.

Figures 3A and B show the results after quantifying the circularity, aspect ratio and size of T47D clusters in monocultures (blue bars) and in co-cultures (red bars) with different ECM compositions. As observed in Figure 2, T47D clusters cultured in CN had a more circular morphology (circularity and aspect ratio closer to 1) in monocultures than in co-cultures (Figure 3A and B; *$p<0.02$), and T47D clusters were more circular in CN than in 100FN (Figure 3A, $p<0.05$). Similarly, T47D clusters were more circular in monocultures than in co-cultures in microenvironments with 100μg/mL of FN (100FN, Figure 3A and B, *$p<0.02$) or 100μg/mL of LN (100LN, Figure 3A and B, *$p<0.02$) However, no significant changes in the circularity and aspect ratio were found between monocultures and co-cultures in ECM compositions containing low FN or LN concentrations (10FN, 10LN, 50LN; Figure 3A and B). Also, monocultures of T47D cells were more circular in CN than monocultures in 10FN or 10LN (Figure 3A and B,

$p<0.05$). Together, these results show that T47D cells became less circular and more elongated when co-cultured with HMF cells in CN, 100FN or 100LN, and suggests that T47D cells also adopt an elongated morphology in 10FN or 10LN even without HMF cells. A possible explanation for this is that T47D monocultures can be producing FN and LN endogenously when cultured in 10FN and 10LN, and mimic the microenvironments of co-cultures in 100FN and 100LN.

Additionally, the size of T47D clusters was examined in monocultures and co-cultures of different ECM compositions. Figure 3C shows percentages of T47D clusters with sizes varying from 369μm^2 to 2583μm^2 in different ECM compositions. An average of 88% of all the T47D cells in monocultures cells grew as small clusters (i.e. 369μm^2 to 1107μm^2), and only 6% of the clusters were big (i.e. 1845μm^2 to 2583μm^2). On the other hand, T47D cells cultured with HMF cells in CN, 50FN, 50LN, and 10FN had a wider distribution of sizes. Interestingly, in 100FN the highest percentage of clusters had small (23%, 738μm^2) and big (23%, 2583μm^2) clusters. In microenvironments containing 10LN, the majority of clusters remained small (79%, 369μm^2 to 1107μm^2), and only 4% of clusters were big (2583 μm^2). However, when LN concentration was increased to 100LN, the population of biggest clusters (2583 μm^2) was increased to 30%, and the population of small clusters (369μm^2 to 1107μm^2) was significantly reduced to 47%. Therefore, T47D cells were more sensitive to changes in the ECM composition when co-cultured with HMF cells and the proliferation of T47D cells appears to be affected by the ECM compositions when co-cultured with HMF cells.

Proliferation of T47D cells in different ECM compositions

Measuring the proliferation of breast cancer cells has shown to be useful for studying stroma-to-carcinoma cell signaling, predicting clinical response, and providing a prognosis indicator. For example, previous studies showed that the overexpression of syndecan-1 (Sdc-1) in fibroblasts promotes breast cancer cell proliferation[13]. In other studies, a decrease in the proliferation index was predictive of good clinical response[14], and the proliferation of tumor cells in conjunction with tumor size, grade, nodal status, and steroid receptor status were useful prognostic indicators[15]. Here, the proliferation of T47D cells in monocultures and co-cultures with HMF cells was analyzed under the influence of different ECM compositions.

The total cytokeratin (CK) staining area of T47D clusters previously showed to correlate with the total number of T47D cells cultured in 3D collagen gels[8, 11]. Therefore, CK staining was used in this work to evaluate T47D proliferation. Figure 4 shows the screening results for monocultures of T47D cells (blue bars) and co-cultures of T47D and HMF cells (red bars) in different ECM compositions. As expected, co-cultures in CN supported a 2.2-fold increase in T47D cell number compared to monocultures in CN (*$p<0.02$, Figure 4), which agreed with previously reported data[8, 11]. In microenvironments containing 50LN or 100LN, no specific trend in T47D growth was found between three separate experiments. However, T47D cells seemed to be more sensitive to compositions containing FN or low concentrations of LN (i.e. 10LN). For example, a significant decrease in CK-positive area was observed between co-cultures in CN and co-cultures in 10FN or 10LN (**$p<0.05$, Figure 4), but no effect was observed between monocultures in CN, and monocultures in 10FN or 10LN. Moreover, at higher FN concentrations (i.e. 50FN versus 100FN), the growth of T47D cells was reduced in both co-cultures ($p<0.02$, Figure 4), and monocultures ($p<0.02$, Figure 4). Therefore, monocultures needed higher doses of FN (i.e. 100FN) than co-cultures (i.e. 10FN) in order to decrease T47D

cell growth significantly. These results suggests that HMF cells might be producing FN endogenously that helps to further decrease the growth of T47D cells in co-cultures. Moreover, it identifies FN as an important ECM protein that could be interfering with paracrine signals between T47D and HMF cells that are necessary for inducing T47D growth.

In conclusion, an automated microfludic platform[8] was used to screen for ECM compositions that influenced the 3D morphology and proliferation of T47D breast carcinoma cells. The small volumes of ECM proteins and cells required for loading each microchannel (approximately 2μL per microchannel) allowed for a cost-effective screening of ECM compositions when compared to the volumes required in traditional 3D cell culture assays (approximately 50μL per well). Using morphology as an endpoint revealed differences in the circularity of T47D clusters cultured in CN, 100FN or 100LN, and no differences when cultured in 10FN, or 10LN. Moreover, differences in T47D cluster size between cells cultured at low (10μg/mL) and high (100μg/mL) FN and LN concentrations suggested that the proliferation of T47D cells was also dependent on the ECM composition. High concentrations of FN (100μg/mL) were needed to decrease the growth of T47D cells in monocultures significantly, but in co-cultures low concentrations of FN (10μg/mL) were sufficient to decrease T47D growth. Together, the screening results show that the ECM composition affects the 3D morphology and proliferation of T47D cells, and suggest that FN might be an important ECM protein that interferes with paracrine signals between T47D and HMF cells that are needed for inducing T47D growth. Therefore, the screening platform presented in this work provides an information-rich *in vitro* assay where cells are cultured in more physiologically relevant 3D microenvironments and promises to be useful for advancing the development of more *in vivo*-

like screening platforms to further study the role of ECM and stromal components, and identifying new drug targets in breast cancer.

ACKNOWLEDGEMENTS

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Figures captions

Figure 1- A) A microchannel array was used to culture T47D cells in monocultures (first 5 microchannels per row) and in co-cultures with human mammary fibroblasts (HMF, last 5 microchannels per row) with different ECM compositions. Microchannels dimensions are shown in millimeters. B) Seven different ECM compositions were used to culture T47D and HMF cells in the microchannel array.

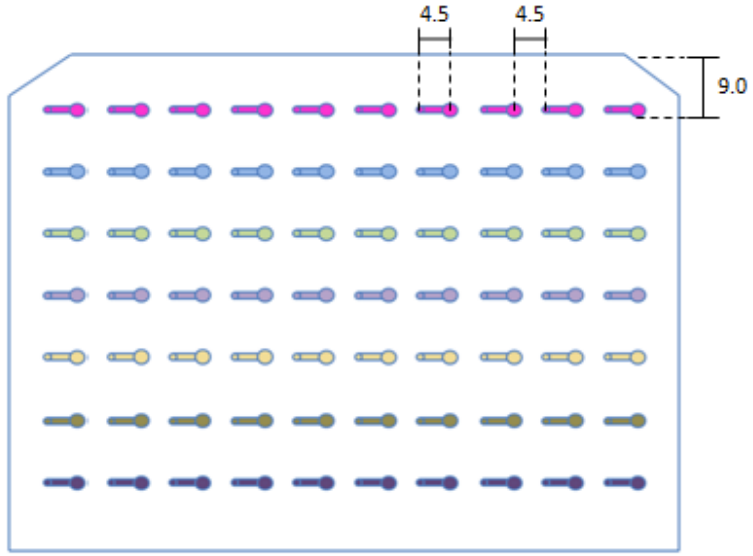
Figure 2- Immunofluorescent images of T47D cells in monocultures and in co-cultures with HMF cells inside microchannels with 1.3mg/mL of collagen (CN), 1.3mg/mL of collagen and 100μg/mL of fibronectin (100FN), and 1.3mg/mL of collagen and 100μg/mL of laminin (100LN). After 7 days of culture, T47D cells were fixed and stained against cytokeratin (red), HMF against vimentin (green), and nuclei with Hoechst dye (blue). Binary images were produced with JeXperiment to analyze the morphology of T47D clusters. Scale bars represent 100μm.

Figure 3- The circularity (A), aspect ratio (B), and size (C) of T47D clusters was examined in monocultures (blue bars) and in co-cultures with HMF cells (red bars) for different ECM compositions after 7 days of culture inside microchannels. The percentage of T47D clusters with sizes varying from 369μm^2 to 2583μm^2 was quantified for monocultures and co-cultures. "Monocultures" represent T47D clusters in all ECM compositions. "CN, 50FN, 50LN", "10FN", "100FN", "10LN", and "100LN" represent T47D clusters in co-cultures. Each chart represents data collected from at least 4 microchannels.

Figure 4- The proliferation of T47D cells was analyzed after 7 days of culture in monocultures cells (blue bars) and co-cultures with HMF cells (red bars). Total CK-staining area represents the

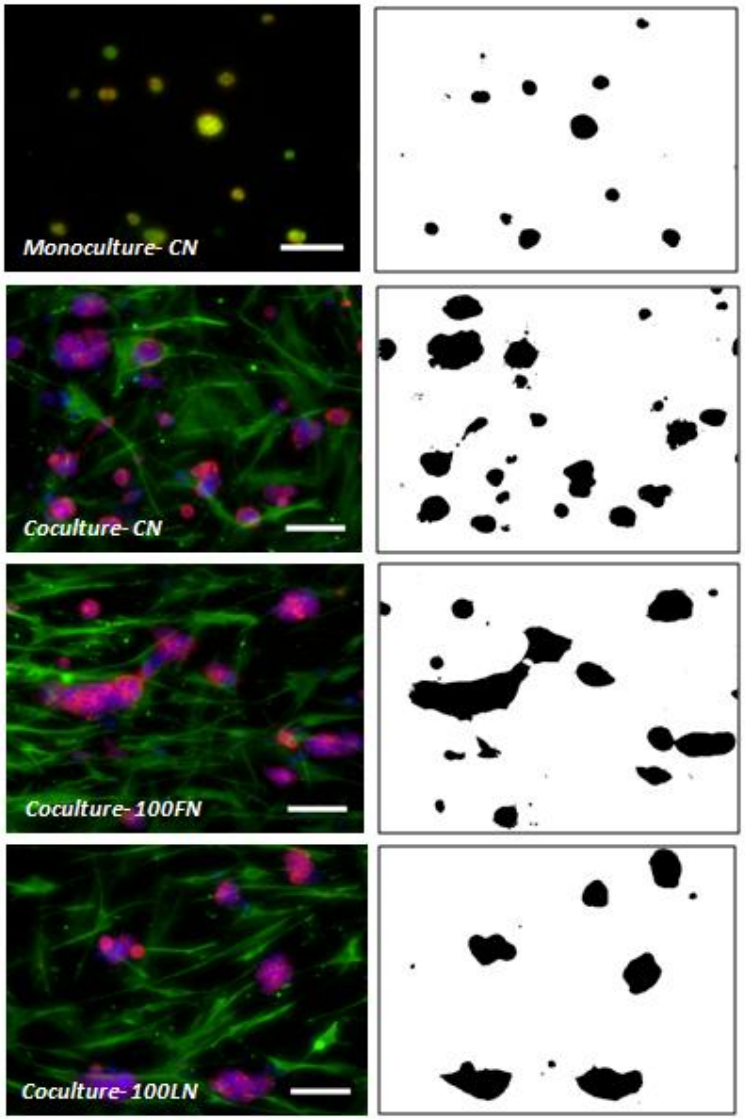
total cell number of T47D cells inside microchannels. n=4, *p<0.02, **p<0.05 compared to CN co-cultures, □ p<0.02, □□ p<0.02.

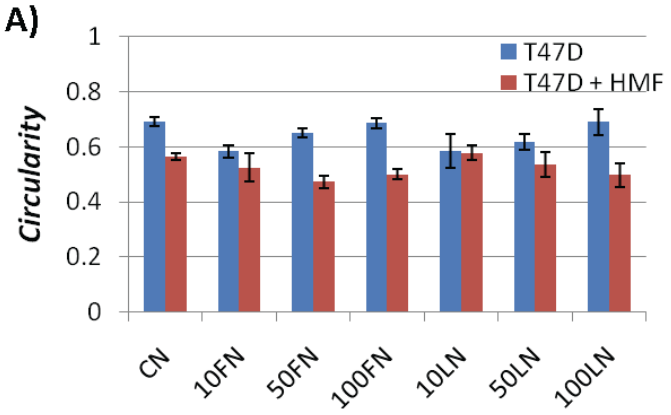
A)

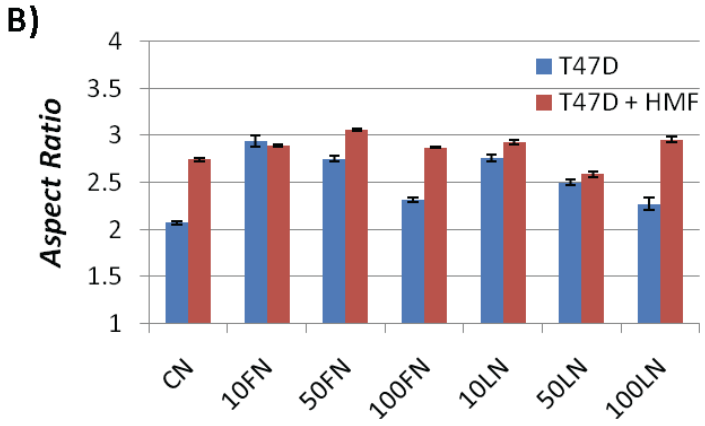


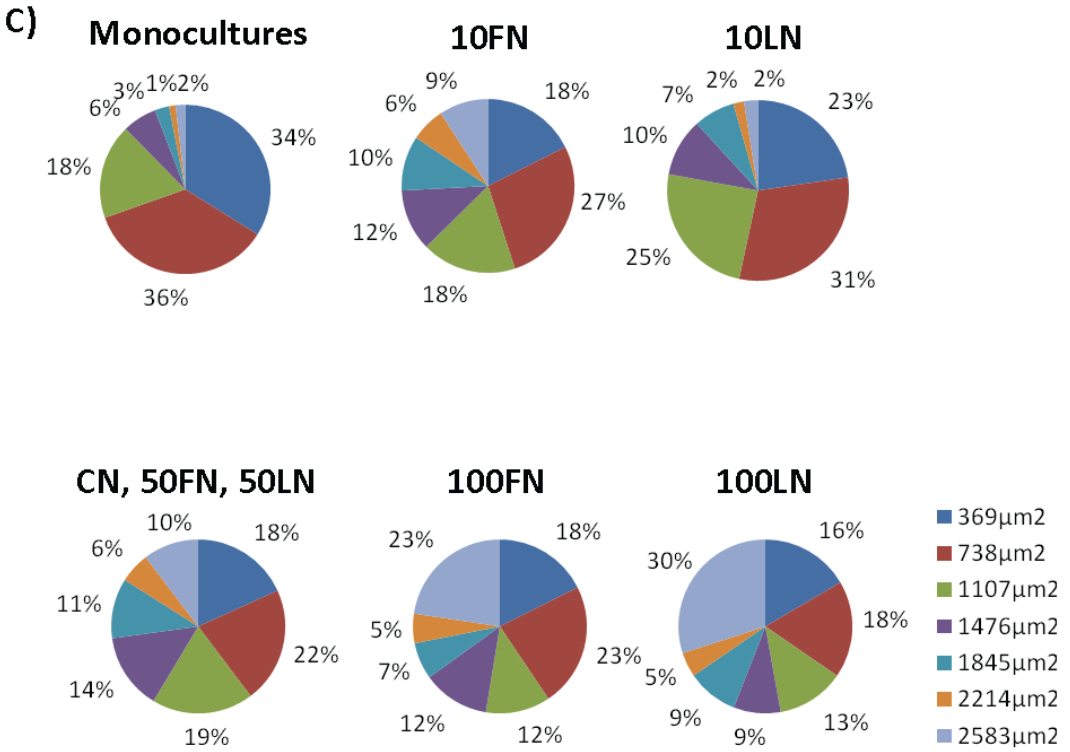
B)

Color	ECM Composition	Abbreviation
	1.3mg/mL collagen type-I	CN
	1.3mg/mL collagen type-I + 10μg/mL fibronectin	10FN
	1.3mg/mL collagen type-I + 50μg/mL fibronectin	50FN
	1.3mg/mL collagen type-I + 100μg/mL fibronectin	100FN
	1.3mg/mL collagen type-I + 10μg/mL laminin	10LN
	1.3mg/mL collagen type-I + 50μg/mL laminin	50LN
	1.3mg/mL collagen type-I + 100μg/mL laminin	100LN


Monoculture- CN
Coculture- CN
Coculture- 100FN
Coculture- 100LN


A)
Circularity
1
0.8
0.6
0.4
0.2
0
T47D
T47D + HMF
CN
10FN
50FN
100FN
10LN
50LN
100LN


B)
Aspect Ratio
4
3.5
3
2.5
2
1.5
1
T47D
T47D + HMF
CN
10FN
50FN
100FN
10LN
50LN
100LN


C)
Monocultures
34%
36%
18%
6%
3%
1%
2%
10FN
18%
27%
18%
12%
10%
6%
9%
10LN
23%
31%
25%
10%
7%
2%
2%
CN, 50FN, 50LN
18%
22%
19%
14%
11%
6%
10%
100FN
18%
23%
12%
7%
5%
23%
100LN
16%
18%
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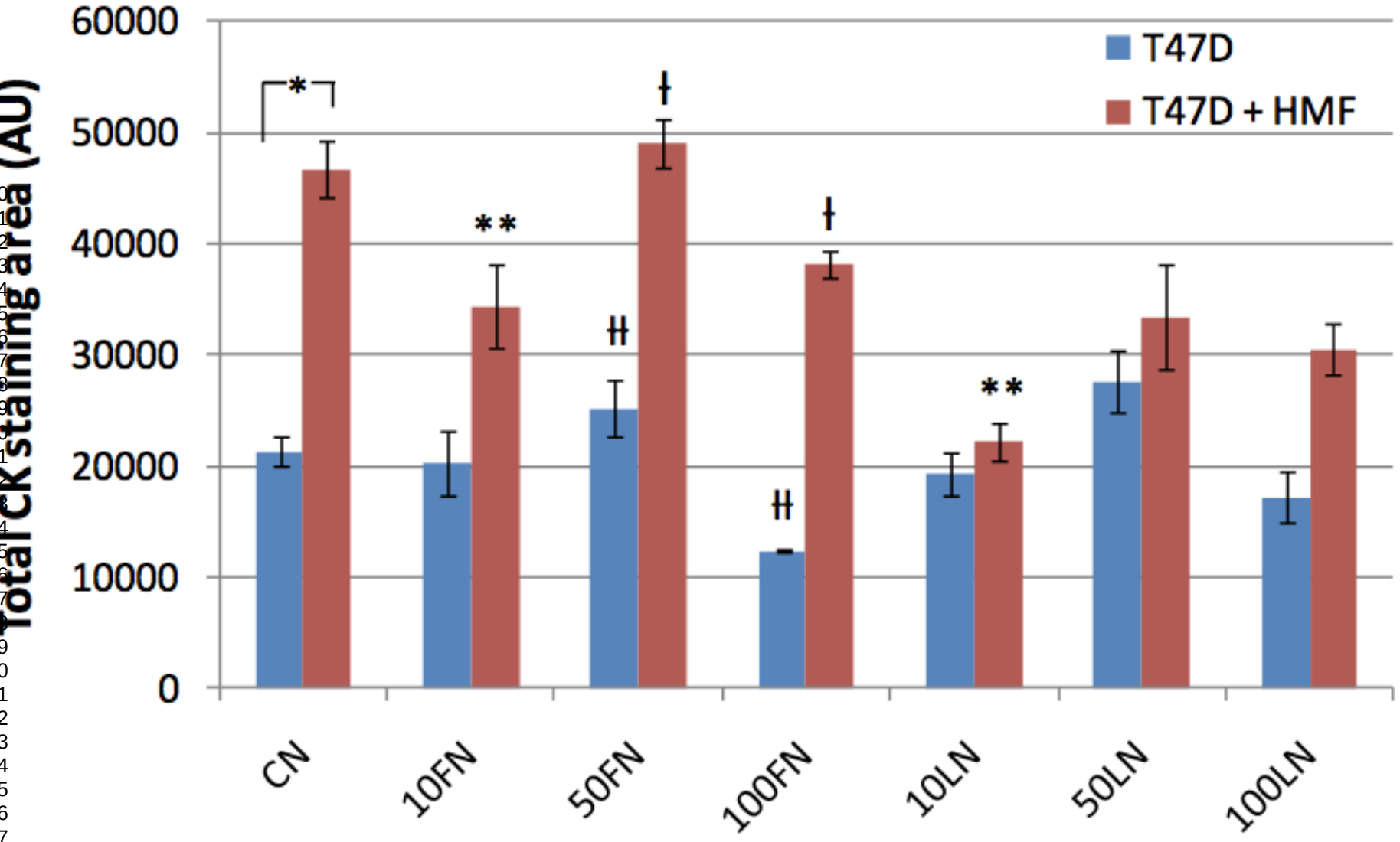
1107µm2
1476µm2
1845µm2
2214µm2
2583µm2


60000
50000
40000
30000
20000
10000
0
Total CK staining area (AU)
T47D
T47D + HMF
*
**
ǂ
ǂ
ǂ
ǂ
**
CN
10FN
50FN
100FN
10LN
50LN
100LN

A micro scale in vitro model of the DCIS-IDC transition: Deciphering the role of the stroma fibroblasts.

Kyung Eun Sung, Carolyn Pehlke, Erwin Berthier, Andreas Friedl, David J. Beebe

Breast cancer progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) is a critical step in breast cancer. We recently developed an efficient 3D microfluidic system that supports the transition from DCIS to IDC. The in vitro system employs microchannels with two inputs and one output enabling MCF10-DCIS.com cells (MCF-DCIS) and human mammary fibroblasts (HMF) to be loaded in two adjacent (side-by-side) compartments. This platform allows investigations of effects of spatial organization on the transition by independently analyzing their morphology and the modifications to the surrounding collagen architecture. Importantly, the compartmentalized platform enables monitoring of both MCF-DCIS and HMF independently including quantitative measures of the collagen architecture associated with each cell type. We observed that the HMF near MCF-DCIS became more protrusive versus HMF relatively far from MCF-DCIS. We have also begun to identify how the HMF become activated and protrusive when co-cultured with MCF-DCIS and to understand the biological function and impact of protrusive HMF during DCIS progression to IDC. We verified that the signaling based on Cathepsin D produced from MCF-DCIS and low-density lipoprotein receptor-related protein-1 (LRP1) form HMF was involved in regulation of the protrusive activity of HMF. Additionally, knocking down LRP1 in HMF inhibited the invasive transition of MCF-DCIS. This study demonstrates one possible route through which MCF-DCIS activate pre-existing fibroblasts and subsequently, leads to the modification of the ECM and the progression to IDC.

250 words limit